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Summary

The current study examined the functions of the Vpr, Vpx, and Nef regulatory proteins of human immunodeficiency viruses. Vpr was found to be important for productive infection of monocytes by HIV-1, but could be replaced by Vpu. Several Vpr mutants have been constructed in viral clones and expression vectors, and a Vpr antibody raised for further structure-function studies of this protein. Studies of Vpx have just been initiated focusing on requirements for its packaging, possible RNA binding, and role in infectivity. An antibody to Vpx is currently being raised. Nef was shown to suppress HIV-1 transcription, and recent gel retardation experiments have identified its activity via a NFkB-like protein. Nef mutants in SIVmac and are HIV-1 are currently being studied.

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TABLE OF CONTENTS

<u>Page</u>	
	<u>Introduction</u>
5	Technical Objectives
6	Hypotheses
6	Background
0b	<u>Body</u>
0b	To define the function of viral protein R (Vpr)
8	To define the function of viral protein X (Vpx)
0	To determine the function and mechanism of action of negative factor (Nef)
6	<u>Conclusions</u>
7	<u>References</u>
8	<u>Appendix - Publications</u>
	1. Structure and Function of Human and Simian Immunosuppressive Virus Nef Proteins
	2. Structure and Function of HIV and SIV Nef Proteins
	3. Identification of a determinant within the human immunodeficiency virus 1 surface envelope glycoprotein critical for productive infection of primary monocytes.
	4. Simian Immunodeficiency Virus Negative Factor Suppresses the Level of Viral mRNA in COS cells
	5. Protein N-myristoylation/AIDS/fatty acid analogs
	6. Functional Analysis of HIV-1 and SIV Nef Proteins
	7. A Determinant within the HIV-1 gp120 Envelope Protein Critical for Infection of Primary Monocytes.

(5) INTRODUCTION - Derived from original Contract Proposal

a) Technical Objectives

1. To define the function of viral protein R (VPR)
 - a. Express HIV-1, HIV-2, and SIV vpr genes in E. coli
 - b. Develop antibodies to recombinant VPR products
 - c. Determine size(s) of VPR products in acute and chronically infected lymphoid and monocytoid cells infected with HIV-1, HIV-2, and SIV
 - d. Determine cellular localization of VPR
 - e. Assess co- and post-translational modifications of the VPR proteins
 - f. Isolate cDNAs encoding VPR
 - g. Determine role of VPR in HIV-1, HIV-2, and SIV replication in a variety of lymphoid and monocytoid cells
 - h. Determine mechanism of action of VPR in enhancing HIV-1 infectivity and/or replication in MT4 lymphoid cells
 - i) Determine effects of VPR on HIV-1 infectivity
 - ii) Determine effects of VPR on HIV-1 production
 - iii) Determine if VPR effects can be complemented in trans
 - iv) Determine if VPR effects are independent of other regulatory proteins
 - v) Determine structure-function relationships of VPR protein
 - i. Determine role of VPR in vivo with appropriate model systems
 - i) Scid/hu mouse models
 - ii) Rabbit model systems
 - iii) SIV model systems in rhesus macaques
 - j. Determine role of VPR in modulating disease in HIV-1 infected humans
2. To determine the function of viral protein X (VPX)
 - a. To assess effects of VPX on replication and cytopathicity of HIV-2 in T lymphoid and monocytoid cells
 - i) To determine effects of VPX on site of virus assembly in the cell
 - b. To determine cellular localization of VPX in HIV-2 infected cells
 - c. To determine if there are co- or post-translational modifications of VPX
 - d. To assess whether VPX proviral mutants can be complemented in trans by a VPX expression clone
 - e. To determine structure-function relationships of VPX
 - f. To assess effects of VPX on replication and cytopathicity of SIV in T lymphoid and monocytoid cells
 - g. To assess role of VPX in vivo with animal model systems
3. To determine function and mechanism of action of NEF
 - a. To determine relative effects of HIV-1 NEF on viral RNA transcription, degradation, and nuclear-cytoplasmic transport
 - b. To characterize NEF responsive sequences
 - c. To characterize mechanism of transcriptional suppression by NEF
 - i) Gel retardation measurements
 - ii) DNA footprinting studies
 - iii) In vitro transcription studies
 - d. To determine role of phosphorylation, GTP binding, GTPase activity, and myristoylation acceptor activity in NEF activity
 - e. To determine effects of NEF on cellular proteins including those which may modulate HIV-1 infectivity or replication
 - f. To determine role of NEF in HIV-2 and SIV replication
 - g. To determine role of NEF in vivo with animal model systems
 - h. To determine role of NEF in modulating manifestations of HIV-1 infection in humans
 - i. To determine therapeutic role of a retrovirus expressing NEF

b) Hypotheses

Regulatory genes of HIV-1, HIV-2, and SIV are important in modulating virus infection and transmission in vivo. Viral proteins R (VPR) and X (VPX) and the negative factor (NEF) are three of the least well characterized regulatory proteins. Identification of their functions, their mechanisms of actions, and structure-function relationships of each protein, in vitro and in vivo will assist in our understanding of the pathogenesis of HIV induced disease. This information will be critical in defining therapeutic approaches to suppressing HIV-1 infection, replication, and transmission.

c) Background

i) Basis

HIV-1 and HIV-2 cause a slowly progressive immunosuppressive disorder in humans. One species of simian immunodeficiency virus (SIV) derived from rhesus macaques, SIV-MAC, can cause a similar disorder in this species of monkeys (Chakrabarti et al., 1987). Related lentiviruses are found in other species of monkeys including mandrills (Tsujimoto et al., 1989), sooty mangabeys (Hirsch et al., 1989), and African green monkeys (Fukasawa et al., 1988). More distantly related lentiviruses causes immunosuppression in cats (feline immunodeficiency virus) (Pederson et al., 1987; Luciw et al., 1989), sheep (visna virus) (Haas et al., 1985), goats (caprine-arthrititis encephalitis virus) (Narayan & Cork, 1985), and horses (equine infectious anemia virus) (Issel et al., 1986).

These viruses are biologically and structurally related. They differ from avian and murine retroviruses in the complex nature of their genomes. In addition to genes encoding structural and enzymatic virion proteins, GAG, POL, and ENV, these viruses all encode a number of regulatory proteins (Haseltine et al., 1988). Seven regulatory proteins have been identified thus far.

TAT is a positive feedback regulator of expression of virion and regulatory proteins, working primarily at the level of transcriptional initiation or elongation, and to a lesser degree at a post-transcriptional level.

REV is a differential regulator that increases expression of virion proteins at the expense of regulatory proteins by increasing the transport of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm and by increasing their stability. In addition, a fusion protein between TAT and REV has recently been described (Felber et al., 1989b); its function is unknown.

VIF is important for the infectivity of the virus particle by a post-translational mechanism that remains to be defined. VPU is important for mature virus assembly at the cell surface. VPR, VPX, and NEF are additional regulatory proteins whose functions will be the focus of this study, and will be discussed below.

Regulatory proteins are likely to be important in determining the level of virus replication at different stages of disease, in determining the types of interaction with the immune system, and in modulating virus infectivity and transmission. A better understanding of their structure, expression, and mechanism of action will undoubtedly improve our understanding of pathogenesis, lead to the development of new diagnostic assays, provide new insights into therapeutic maneuvers which may suppress virus replication and/or cytopathicity, and assist in the development of a vaccine for HIV prevention.

Viral Protein R (VPR)

The vpr gene is found in the genomes of HIV-1, HIV-2, simian immunodeficiency virus (SIV) of rhesus macaques (SIV-MAC), SIV of sooty mangabeys (SIV-SM), but not SIV of African green monkeys (SIV-AGM), or SIV of mandrills (SIV-MN) (Wong-Staal et al., 1987; Guyader et al., 1987; Chakrabarti et al., 1987; Fukasawa et al., 1987; Tsujimoto et al., 1989; Hirsch et al., 1989). An open reading frame is also found in a similar position in the visna virus genome (Sonigo et al., 1985). The conservation of the predicted VPR proteins among these different lentiviruses is almost as great as that of GAG and POL proteins.

The HIV-1 VPR protein is 78 amino acids long in several strains, and 96 amino acids long in the remaining strains (Meyers et al., 1989). Functional proviral clones of HIV-1 have been identified with either form of the vpr gene (Dedera et al., 1989; Adachi et al., 1989). Among the first 70 amino acids, 50% conservation of amino acid sequences are noted (Meyers et al., 1989). The HIV-2 VPR protein is 105 amino acids in length, whereas that of SIV-MAC is 97 amino acids long (Guyader et al., 1987; Chakrabarti et al., 1987).

The VPR proteins of HIV-1, HIV-2, and SIV-MAC are expressed *in vivo* as evidenced by the presence of antibodies reactive with recombinant VPR products in 33-67% of infected humans or rhesus macaques (Wong-Staal et al., 1987; Lange et al., 1989; Yu et al., 1989). A single antibody to the 96 amino acid form of the HIV-1 VPR product has been developed and claimed to detect a 13 kd VPR protein in cells acutely infected with HIV-1 (Lange et al., 1989). The poor quality of the radioimmunoprecipitation analyses using this antibody suggest that the specificity and avidity of this antibody are poor. An antibody to the SIV-MAC VPR product has also recently been developed (Yu et al., 1989), but results with this antiserum have not yet been reported. No antibody to the HIV-2 VPR product has yet been developed.

Work from our laboratory has demonstrated that the HIV-1 and HIV-2 VPR products are dispensable for virus infectivity, replication, and cytopathicity (Dedera et al., 1989). Proviral clones have been constructed expressing a 2 (R2), 22 (R22), 31 (R31), 40 (R40), 78 (R78 or X), or 96 (R96) amino acid form of the VPR product. No differences in the above noted parameters were detected in H9, MOLT 3, CEM, U937, or SUP T1 cell lines, or peripheral blood lymphocytes. HIV-2 proviral clones have been constructed which express either a 105 (MR105 or SE) or 6 amino acid (MR7) form of VPR. No alterations in infectivity, replication, or cytopathicity were noted with viruses derived from these clones in H9, MOLT 3, CEM, SUP T1, Jurkat, or U937 cell lines, or primary human lymphocytes or monocytes.

However, recent data suggests a cell-type dependent effect of vpr expression or action. In MT4 cells, the kinetics or replication of virus derived from R2 were significantly different from that of R78, with retarded and diminished virus yield from the vpr mutant. This finding has now been obtained in 6 replicate experiments with 3 different preparations of R2 and R78. Cytopathicity was comparably depressed. The novel feature of this cell line which may account for this effect is unknown; there may be a relationship to human T-lymphotropic virus type 1 (HTLV-I) expression in MT4 cells.

In addition, we have noted subtle morphological differences of virus derived from vpr mutant infected cell lines compared to those infected with the parental virus. R2 virus particles appeared to be less homogenous and more immature than those derived from R78. This may suggest an effect of VPR in virus assembly or maturation. Possible alterations in the structure of the virus particle could account for possible changes in infectivity of the virus.

Viral Protein X (VPX)

The vpx gene is found in HIV-2, SIV-MAC, SIV-AGM, SIV-SM, but not HIV-1 or SIV-MN (Guyader et al., 1987; Chakrabarti et al., 1987; Fukasawa et al., 1988; Hirsch et al., 1989; Ratner et al., 1985a; Tsujimoto et al., 1989). It is an immunogenic protein expressed in vivo, to which 85% of HIV-2 infected humans and 20% of SIV-MAC infected rhesus macaques generate antibodies (Kappes et al., 1988; Yu et al., 1988).

The VPX product is a 112 amino acid proline-rich protein which is found in the virion in equimolar ratio to the GAG capsid (CA; p24) antigen (Henderson et al., 1988). It has also been found to be a nucleic acid binding protein, though specificity for this property remains to be investigated.

Four groups of investigators, including our own group, have now reported on findings of SIV or HIV-2 viruses with alterations in vpx (Yu et al., 1988; Guyader et al., 1989; Hu et al., 1989; Kappes et al., 1989). All groups agree that VPX is dispensable for virus infectivity, replication, and cytopathicity. For example, we have found no effect of VPX on replication of HIV-2 in CEM, H9, U937, SUP T1, and Jurkat cell lines. These studies were carried out with HIV-1 proviral clones capable of coding for the full-length VPX protein, or a clone with a serine substitution for the initiator methionine (MX1), a clone with a termination codon at position 22 (MX22), and a clone with the same mutation present in MX1 as well as a frameshift mutation at position 62 and another termination codon at position 70 (MX1+62).

However, divergent results were obtained in studies of vpx mutant replication on primary T lymphocytes. Whereas Guyader reported a 10-fold decrease in HIV-2 replication in the absence of VPX on T lymphocytes, our own studies have repeatedly failed to detect an alteration in infectivity, replication, or cytopathicity of HIV-2 viruses in primary human lymphocytes with or without vpx (Guyader et al., 1989; Hu et al., 1989). A more comprehensive examination of kinetics of HIV-2 and SIV replication in primary human and macaque lymphocytes and in primary monocytes is clearly indicated to resolve these potentially important discrepancies.

Our group has recently detected a particularly intriguing property of VPX to direct HIV-2 budding to particular sites in the cell. In the presence of VPX, HIV-2 was found in H9 cells to bud intracellularly and at the plasma membrane. The morphology of the virus particles was generally mature and rather homogeneous. In the absence of VPX, HIV-2 buds exclusively at the plasma membrane. The virus particles were generally less mature and less homogeneous.

Negative factor (NEF)

The nef gene, unlike the vpr and vpx genes, is poorly conserved between different strains of HIV-1 and other lentiviruses (Ratner et al., 1985;

Meyers et al., 1989). A similar open reading frame, however, has been described also in HIV-2, SIV-MAC, SIV-AGM, SIV-MN, and SIV-SM (Guyader et al., 1987; Chakrabarti et al., 1987; Fukasawa et al., 1987; Tsujimoto et al., 1989; Hirsch et al., 1989).

The NEF protein is immunogenic in vivo in infected humans. Both humoral and cell-mediated immune responses have been detected to this protein (Allan et al., 1985; Arya et al., 1986; Franchini et al., 1986 and 1987). Perhaps the most intriguing finding is the identification by several different investigators of antibodies reactive with NEF early after infection and frequently prior to the detection of other anti-HIV-1 antibodies (Ameisen et al., 1989a and b; Sabatier, et al., 1989; Reiss, et al., 1989; Ronde et al., 1989; Chengsong-Popov et al., 1989; Laure et al., 1989). This

suggests that NEF may be the first viral protein to be expressed in vivo. Recent data from tissue culture experiments confirm that NEF mRNA is expressed after infection earlier than mRNAs for other regulatory and structural proteins (Klotman et al., 1989).

Five laboratories, including our own, have now reported that NEF is a negative regulator of virus replication (Luciw et al., 1986; Terwilliger et al., 1986; Ahmad & Venketassen et al., 1988; Niederman et al., 1989 & Levy et al., 1989). However, one laboratory has failed to

detect an effect of NEF (Kim & Baltimore, 1989). Differences in sequence of the NEF product expressed or other technical difficulties may explain the discrepancy. Differences in NEF expression, NEF action, or NEF responsiveness have been demonstrated for different HIV-1 clones (Levy et al., 1989). Kim & Baltimore have not yet carried out similar experiments with proviral clones obtained from any of the five laboratories which have described down-regulatory effects of NEF.

In studies of SIV-MAC clones, all of those which are capable of giving rise to virus in macaque lymphocytes have a defect in nef, whereas the single clone which is not functional has an intact nef gene (Desrosiers, personal communication).

Our own laboratory has recently localized the effect of NEF to an effect on viral RNA levels (Niederman et al., 1989). This effect is at least partially due to an effect on viral transcription as determined by nuclear run-off experiments. Effects on RNA transport or degradation have not been excluded. Confirmation of these results was reported by Ahmad & Venketessan (1988).

The NEF protein has also been reported to down-regulate human CD4 expression (Guy et al., 1987). However, this study was carried out with vaccinia expressed NEF, and only a single control experiment was done examining another lymphocyte surface antigen, 4B4, which was only minimally down-regulated. However, differences in stability of different antigens on the surface of lymphocytes could be reflected in vaccinia infected cells, and the effect may not be specific to NEF. Further studies of the effects of NEF on cellular protein expression and growth are warranted.

Nef is expressed as two proteins from the same mRNA due to utilization of different AUG codons (Ahmad & Venketessan, 1988). When the first AUG codon is recognized a 206 amino acid, N-myristoylated 27 kd protein is expressed. This protein may be phosphorylated at the threonine at position 15 by protein kinase C (Guy et al., 1988). A second NEF product is expressed from utilization of the second AUG codon to produce a 187 amino acid, 25 kd protein (Ahmad & Venketessan, 1988). Both proteins can bind and cleave GTP, and can autophosphorylate at a carboxyl terminal serine residue in the presence of GTP (Guy et al., 1988). Sequence similarities between amino acids 95 and 111 of NEF and other nucleotide binding proteins are readily apparent (Samuel et al., 1987; Guy et al., 1988). The possible relationships of these interesting biochemical activities with NEF activity remain to be investigated.

(6) BODY

1) To define the function of viral protein R (VPR)

a) Express HIV-1, HIV-2 and SIV vpr genes in E. coli

In the original application we had proposed to express each form of vpr in E. coli, in order to obtain protein that could be used for immunization of rabbits for antibody development. We have essentially bypassed this undertaking, by obtaining a purified synthetic HIV-1 Vpr protein from Dr. Gras-Masse. Furthermore, the current availability of antisera to the HIV-2 and SIVmac Vpr proteins (kindly provided by Dr. T.H. Lee, Harvard) also precludes the requirement for the expression of these proteins in E. coli for this purpose.

b) Develop antibodies to recombinant Vpr products

We have inoculated a single rabbit with the synthetic HIV-1 Vpr protein and have now obtained over 100 ml of antisera over the last six months. We have compared our antisera to those provided by Dr. T.H. Lee, AIDS Repository, and Dr. George Shaw (U. of Alabama), and find significantly better reactivity by radioimmunoprecipitation analysis with our antiserum towards either [3H]-leucine labeled protein expressed in reticulocyte lysates or in transfected BSC40 cells (see section 1.i.v).

We have infected CEM cells with HIV-2 strains with or without mutations in vpr, and will use lysates from these cells to test the reactivity of the anti-HIV-2 Vpr antiserum. We will also examine the reactivity of this antiserum with HIV-2 Vpr expressed in reticulocyte lysates and in transfected BSC40 cells (see section 2.c).

c) Determine the size(s) of Vpr products in acute and chronically infected lymphoid and monocytoïd cells infected with HIV-1, HIV-2, and SIV

Cell lines expressing HIV-1 and HIV-2 Vpr proteins during acute or chronic infection are currently being prepared for this purpose. In each case, we will compare the proteins synthesized in infected cells to those synthesized in vitro in reticulocyte lysates. This will be useful in determining whether Vpr is initiated or terminated at the same sites in infected cells, whether proteolytic processing occurs, and whether other cellular or viral proteins co-immunoprecipitate with Vpr.

d) Determine the cellular localization of Vpr

We have begun initial studies to characterize the cellular localization of Vpr. For this purpose, we have utilized clone pTM3 into which is cloned the HIV-1 vpr gene. This clone allows expression in vitro in reticulocyte lysates after transcription with T7 polymerase. In addition, transfection of this clone into tissue culture cells allows expression after infection with a recombinant

vaccinia virus (VV) expressing T7 polymerase (VVT7). We have succeeded in detection of [3H]-leucine labeled Vpr protein in BSC40 cells using this technique. Experiments utilizing biochemical fractionation by ultracentrifugation are underway to assess cellular location of Vpr. We will also assess cellular localization using a Vpr expression clone with an SV40 promoter to rule out the possibility that VV infection perturbs cellular localization. In addition, we will assess Vpr localization in HIV-1 infected cells. Each mutant form of Vpr will be examined by the same techniques. Similar experiments will be performed with the HIV-2 Vpr expression clones as well. Lastly, we will also examine Vpr by immunofluorescence localization by confocal microscopy and immunogold electron microscopy. The immunofluorescent experiments will be initiated shortly. A visiting scientist with extensive experience in immunolocalization electron microscopic techniques, Dr. Jiang Wang will join our group in March, 1992 to perform the latter experiments.

e) Assess co- and post-translational modification of the Vpr protein

Experiments to examine whether HIV-1 or HIV-2 Vpr proteins are phosphorylated, O-glycosylated, sulfated, or palmitoylated will be initiated within the next 2-3 mos.

f) Isolate cDNA encoding Vpr

The experiments on isolation of Vpr cDNAs are being carried out jointly with investigators on contract DAMD17-90C-0057. For this purpose, primers have already been synthesized, and are currently being tested. Such cDNAs will be useful for two purposes. First, we will determine if there are mRNAs which may encode fused proteins with the Vpr open reading frame and some other HIV-1 open reading frame. Second, we will examine sequence heterogeneity and correlate it with functional differences in vpr sequences obtained from patients at different stages of disease.

g) Determine role of Vpr in HIV-1, HIV-2, and SIV replication in a variety of lymphoid and monocytoïd cells

We have pursued in depth the role of HIV-1 Vpr in regulating infection in primary human monocytes. Interactions with envelope determinants were of great interest. For this purpose, we have cloned a 3' fragment of a monocyte-tropic clone of HIV-1, ADA, obtained in collaboration with Drs. Howard Gendelman and Monte Meltzer (WRAIR) (Fig. 1). We sought to identify sequences in this clone which would confer upon a non-monocyte-tropic clone (either NL4-3 or HXB2) the ability to infect and replicate in primary human monocytes. The sequences cloned from the ADA isolate included 3.5 kb sequences with vpr, env, nef, U3 LTR, and partial sequences for tat, rev, and the R region of the LTR (shown as shaded boxes in Fig. 1). The entire fragment or portions of it were cloned in place of the corresponding sequences of HXB2 (shown as open box in Fig. 1).

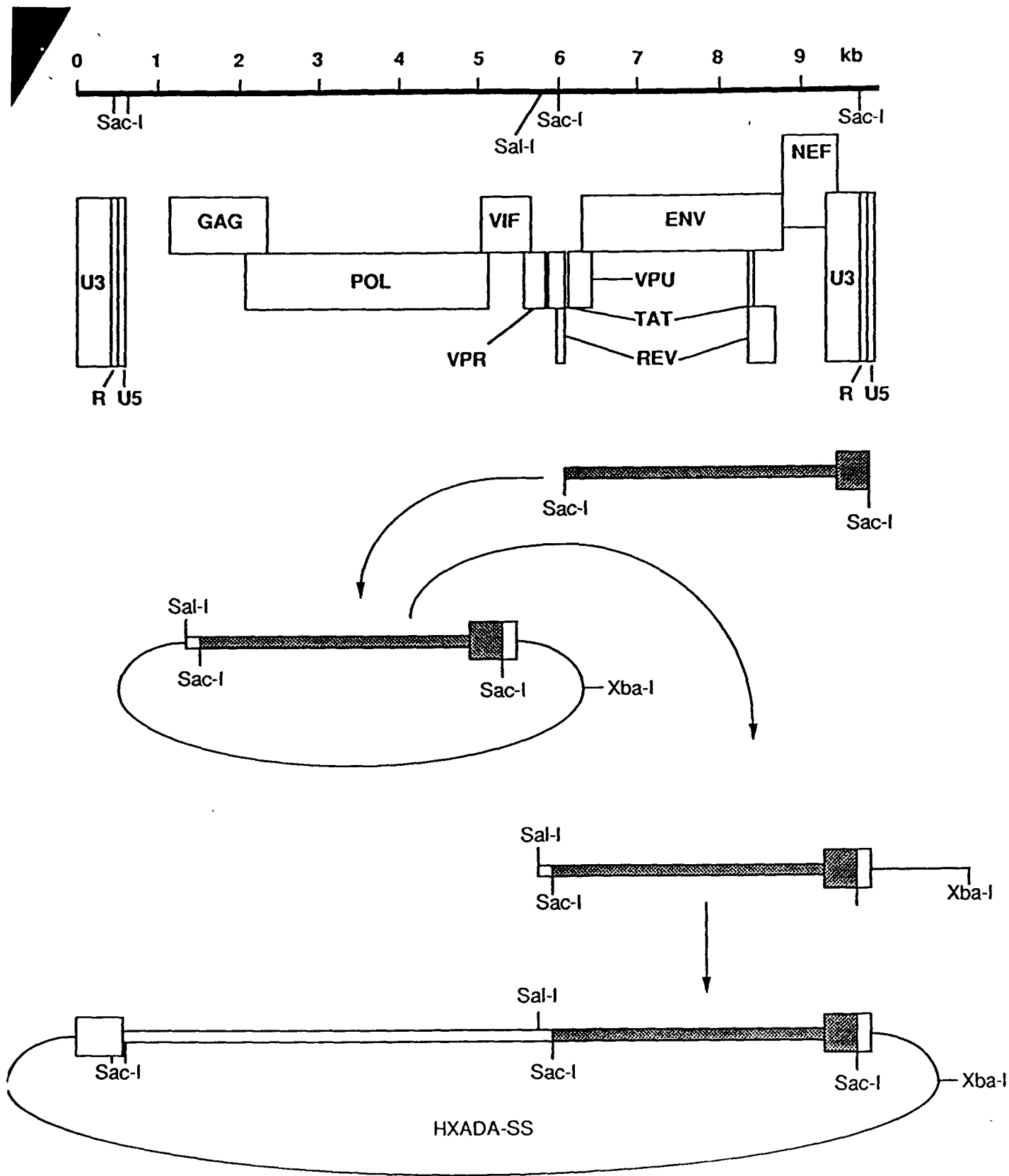


Fig. 1

Upon transfection of these chimeric clones into COS-7 cells or SW480 cells virus was produced in each case which was infectious for primary human lymphocytes. No significant difference (i.e. 3-fold differences) were noted in their ability to replicate in lymphocytes. In contrast, none of the clones gave rise to detectable reverse transcriptase activity (Fig. 2) or p24 antigen expression (not shown) when placed on primary human monocytes in the presence of 500 U/ml M-CSF. In contrast, virus from COS-7 or SW480 cells transfected with the YU-2 clone (kindly provided by G. Shaw, U. of Alabama), obtained from brain tissue without in vitro cultivation, gave rise to high levels of virus production on primary monocytes. This replication phenotype was designated "productive infection."

Cocultivation of monocytes with lymphocytes was performed to identify whether some monocyte cultures exhibited low levels of HIV-1 expression or abortive HIV-1 infection (Fig. 2). Monocytes exposed to HXB2 virus or virus from certain proviral clones with HXB2 and ADA sequences, when cocultivated with lymphocytes, demonstrated no rescue of virus. This phenotype is designated "no infection." However, monocytes infected with virus from a subset of the chimeric proviral clones with HXB2 and ADA sequences, were capable of producing virus after cocultivation with primary lymphocytes. This phenotype of monocyte infection was designated "silent infection."

Sequences in the ADA isolate necessary for conferring monocyte-tropism were identified by analyzing a number of different proviral clones (Fig. 3). All proviral clones gave rise to virus capable infecting primary lymphocytes (PBL), but none were capable of productive infection of monocytes. Clone SM includes ADA vpu and gp120-encoding env sequences, and was capable of silent infection of monocytes. Similar findings were found with clones KM, GG, and GP (representing the 5' half of the GG fragment). Thus, we were able to identify the V3 loop region of envelope as important for monocyte-tropism, and exclude an essential role in silent infection of nef, vpu, tat, rev, or LTR sequences. We independently demonstrated with a number of LTR-CAT constructions that monocyte-tropism did not relate the transcriptional activity of different LTRs in this cellular environment.

To determine, why HXADA-GG gave rise to silent infection but not productive infection, a new set of chimeric proviral clones were constructed. In this case, 5' and 3' sequences of NL4-3 were utilized in place of those of HXB2 (Fig. 4). The rationale for this approach was based on the finding that HXB2 encodes a defective nef product and a defective vpr product. Furthermore, NL4-3 gave rise to no infection of monocytes, even after cocultivation with primary lymphocytes. These chimeric provirus designated NLHXADA clones gave rise to productive infection if the GP fragment from ADA was present.

The monocyte-tropism determinant in gp120 is shown in Fig. 5 in black boxes, including residues 240-333. This region includes the V3 principal neutralizing domain of gp120. Sequences shown as shaded residues represent those which have been shown in mutagenesis experiments to be important for CD4 binding, and largely map 3' to the tropism determinant.

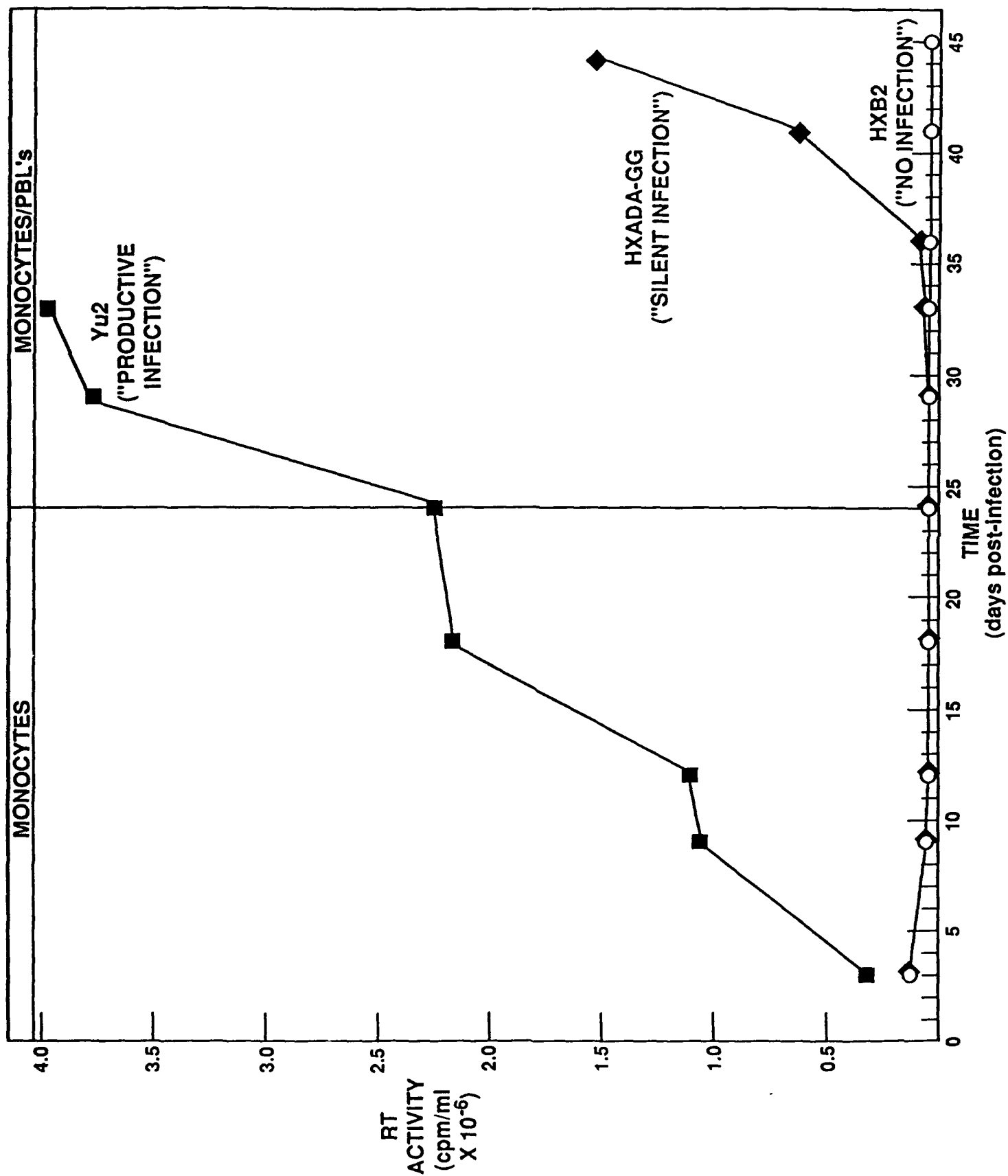


Fig. 2

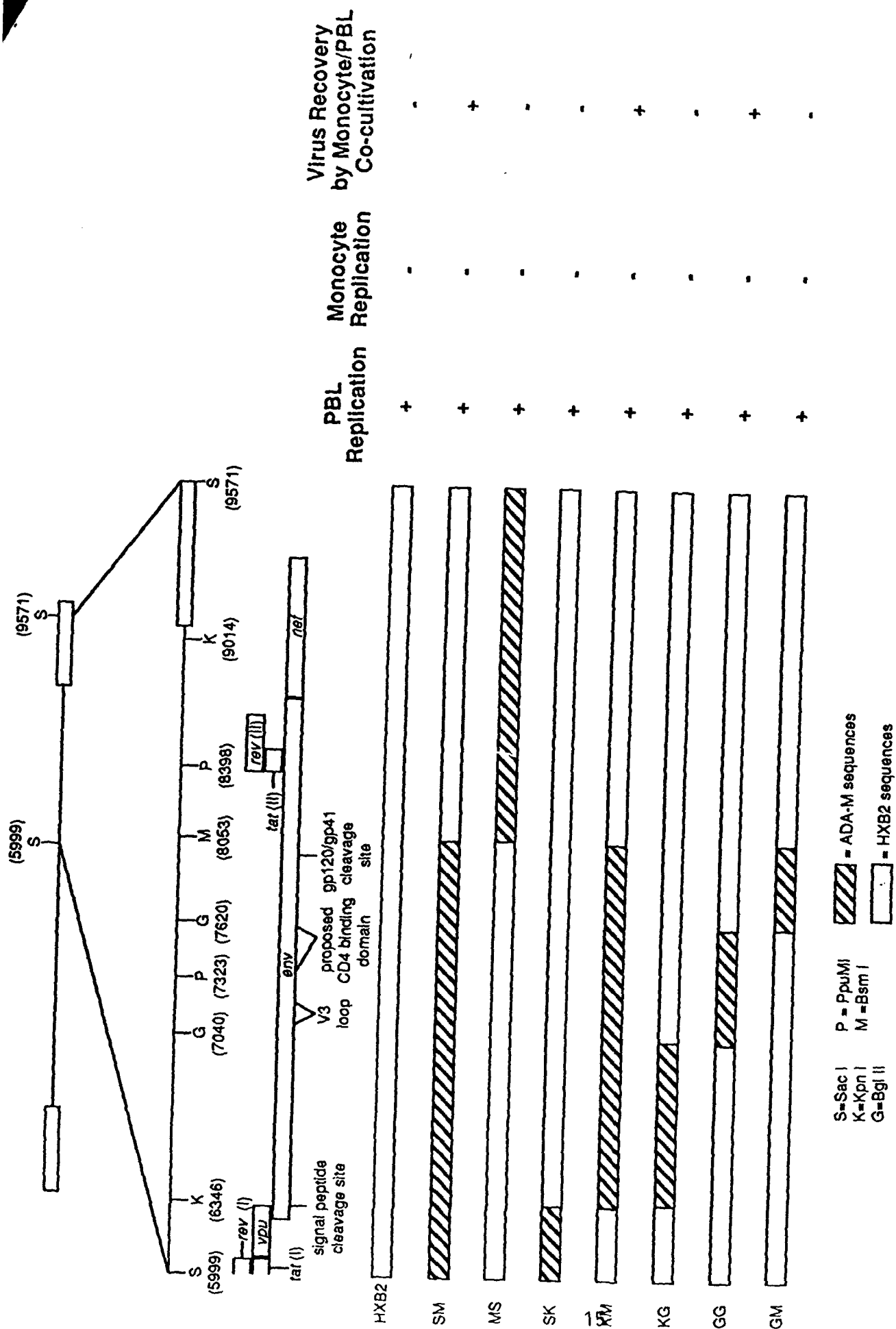
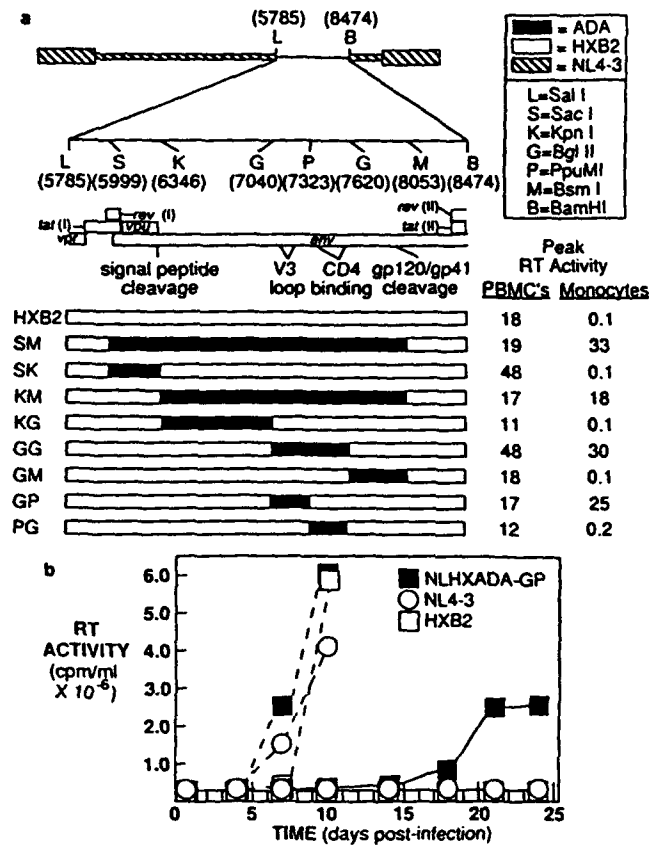


Fig. 3



Replication of NLHXADA recombinant clones in PBMCs and primary monocytes. (a) A panel of recombinant clones generated by exchange of restriction fragments between the ADA, HXB2, and NL4-3 clones is represented diagrammatically on the left. The 2.7-kb *Sal* I/*Bam* HI fragment of each clone has been expanded to demonstrate the relative positions of ADA-derived sequences and HXB2-derived sequences with respect to the open reading frames and restriction enzyme sites shown above. Peak RT activities ($\times 10^{-5}$) generated by virions derived from each clone in PBMCs (days 12-18) and primary monocytes (days 18-24) are indicated on the right. (b) The replication kinetics of NL4-3, HXB2, and NLHXADA-GP in PBMCs (dashed lines) and primary monocytes (solid lines) are shown. Similar results were obtained in two to five replicate experiments.

Fig. 4

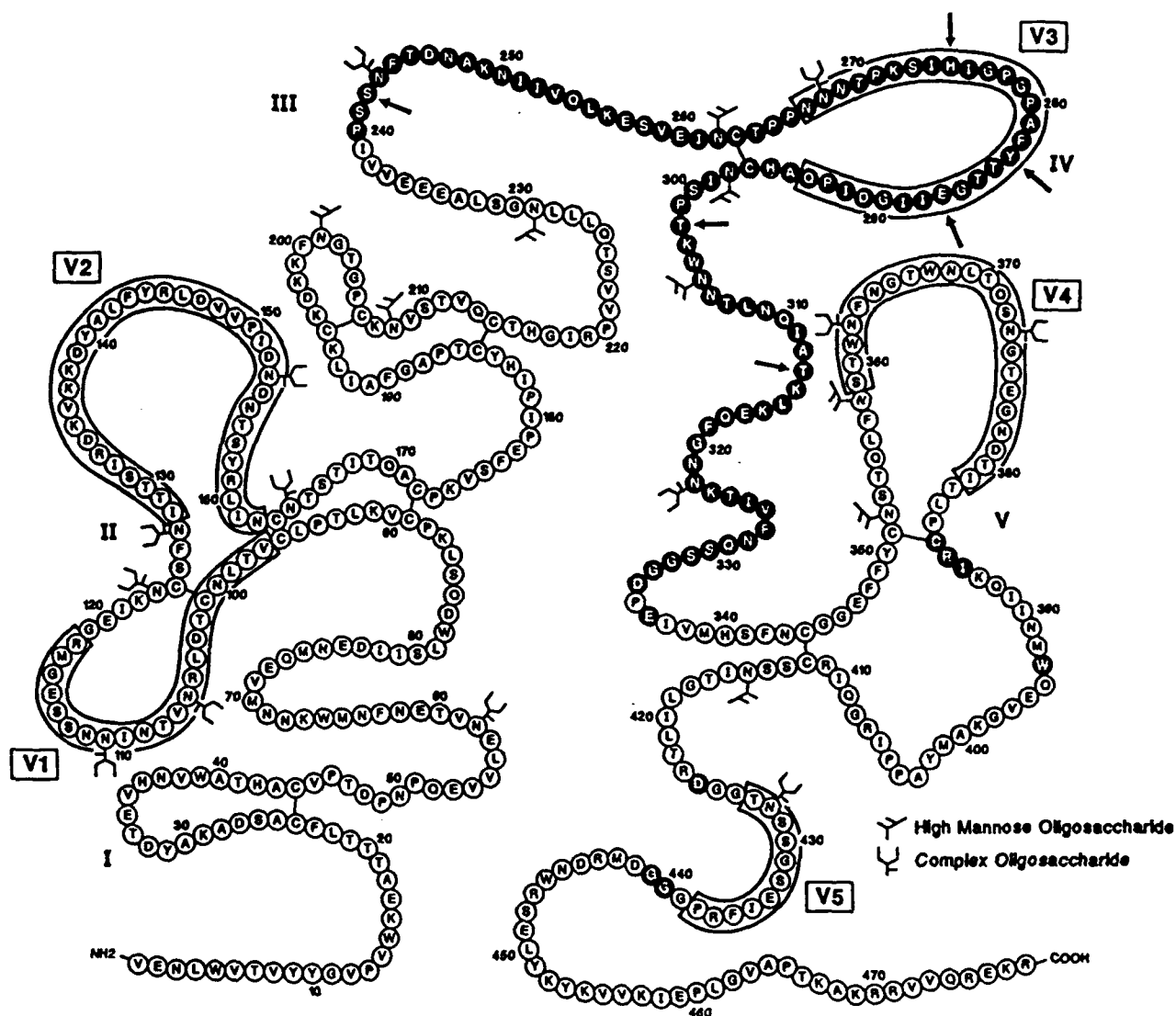


Diagram of the ADA-encoded surface envelope glycoprotein. The primary structure of the mature gp120 protein predicted by the ADA clone is shown superimposed on the secondary structure determination of the HXB2 gp120 [modified with permission (20); copyright The American Society for Biochemistry and Molecular Biology 1990]. Residues encoded by the monocyte tropism determinant (residues 240-333) are represented as shaded circles; residues implicated in CD4 binding are represented as partially shaded circles; the remaining residues are represented as open circles. Residues located within variable domains are enclosed in boxes. Arrows designate residues identified by sequence alignment (see Fig. 4) as potentially involved in conferring the ability to establish productive infection of primary monocytes.

Fig. 5

Sequence alignments of residues 240-333 of isolates which are incapable (HXB2, SF2, NL4-3) or capable of infecting monocytes (ADA, JF-L, YU-2, SF-162) are shown in Fig. 6. Over this 94 amino acid domain, there are 21 amino acid differences between the HXB2 and ADA sequences. Other amino acid differences between the different HIV-1 strains are indicated. Of particular interest is the finding that at only 6 positions (shown in black boxes) are the sequences of monocyte-tropic clones consistently different from those of non-monocyte-tropic clones. Of particular note, residue 283 is always a tyrosine in the case of monocyte-tropic clones. At residue 287, an acidic amino acid was noted in all monocyte-tropic clones, and a basic amino acid in the case of all non-monocyte-tropic clones. These alignments suggest particular amino acids in envelope that can be examined in more detail for their role in monocyte-tropism.

The next set of experiments sought to explain why NLHXADA-GP clone gave rise to productive infection whereas the HXADA-GP clone gave rise to silent infection. We found that a frameshift mutation at codon 65 of the 96 codon vpr open reading frame of clone NLHXADA-GP reverted the phenotype to silent infection (Fig. 7). This strongly suggested that vpr was critical for productive infection of monocytes when the monocyte-tropism envelope determinant was also present. An intact vpr gene without the envelope tropism determinant (as in clone NL4-3) does not give rise to infection of monocytes.

However, the requirement for vpr for productive infection of monocytes was abrogated if ADA sequences from the SK fragment were present (Fig. 7 and 8). Sequences encoded by this SK fragment are shown in Fig. 9 and include the entire vpu gene and small portions of tat, rev, and env genes. The most notable difference between ADA and HXB2 sequences in this domain is the finding that in HXB2 an initiator methionine codon for vpu is not present, whereas in ADA an initiator methionine codon is present. This would suggest that either vpu or vpr together with the envelope determinant allows productive infection of monocytes.

In collaboration with Drs. M. Becich and J. Heuser (Washington University), H. Gendelman (WRAIR), and J. Orenstein (George Washington University), we examined micrographs of monocytes infected with either the non-monocyte tropic isolate NLHXADA-SK (Fig. 10a), the monocyte-tropic isolate with envelope, vpu, and vpr determinants NLHXADA-SM (Fig. 10b), or the monocyte-tropic isolate with envelope and vpr determinants, NLHXADA-GG (Fig. 10c). The presence of giant multinucleated cells by phase microscopy is an obvious characteristic of infected monocytes. Electron micrographic examination of the cells infected with monocyte-tropic clones with or without vpu demonstrated budding mature and immature particles at both the cell surface and in vacuoles. Vpu has previously been implicated in the maturation of virus particles in lymphocytes and budding of particles at the cell surface. A similar effect of vpu in infected monocytes was not apparent. Thus, the function of vpu in monocytes may be different from that characterized in T lymphocytes. Similar findings were found in the presence or absence of vpu, examining virus particles budding from the cell surface by freeze-fracture electron microscopy (Fig. 10e).

Macrophage infection	Clone	----- V3 -----																			↓↓
		242	275										283	287	302				313		
-	HXB2	RS	V	MFTD	NAKTIIVQLNTSVEINCTRPNNTRKRI	R	IQRGPGRAF	V	TIG	K	IGNM	ROAHCHISR	A	KWNNTLKQIA	S	KLREQFGNNKTIIFKQSSGG					
-	SF2	D	N		E A	S	Y	--	H	T	R	IGDI	K	Q	E	V	K		V	N	
-	NL4-3	A			Q	S									A						
+	ADA-M	S		N	KE	S	H	--	Y	T	E	IGDI		T	N	T	K		V	N	
?	JF-L	E	M		K IA	S	T	L--	Y	T	D	IGDI	L	V	Q	V	I	-	V		
+	YU-2	E	N		E V	S	N	--	L	Y	T	E	IGDI	L	K	T	Q	E	E	NP	
+	SF162	E			KE	S	T	--	Y	A	T	IGDI	G	E		V	T	QA	-	V	

Amino acid alignment of proposed tropism determinants from monocyte-nonreplicative and -replicative clones. The predicted amino acid sequence of the monocyte tropism determinant from ADA is aligned with those of six additional clones. The clones SF162, Yu2, and JF-L are grouped with ADA as capable of productively infecting monocytes, whereas the clones HXB2, NL4-3, and SF2 are grouped as incapable of productive monocyte infection, based on the data in Fig. 2 (NL4-3, HXB2, and ADA), on published data (SF2 and SF162; ref. 25), and on our unpublished data (Yu2 and JF-L). Amino acid numbers shown correspond to those of the mature ADA gp120 protein (HXB2 gp120 residues 243-337). Dots mark the position of every 10th residue. Asparagine residues involved in N-linked glycosylation are underlined (20). Arrows indicate residues shown to be necessary for CD4 binding. The V3 domain is also indicated. Positions at which all four monocyte-replicative clones differ from residues encoded by the three monocyte-nonreplicative clones are highlighted by solid boxes.

Fig. 6

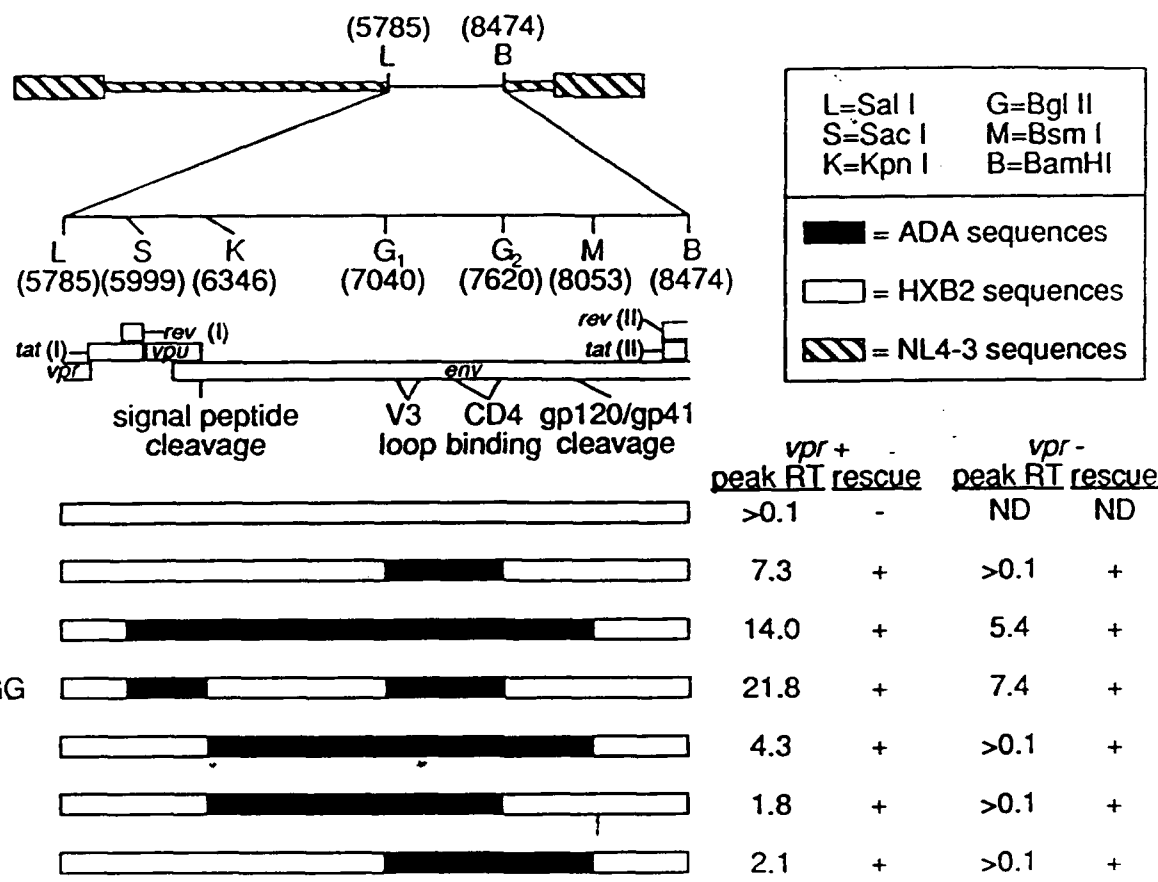


Fig. 7

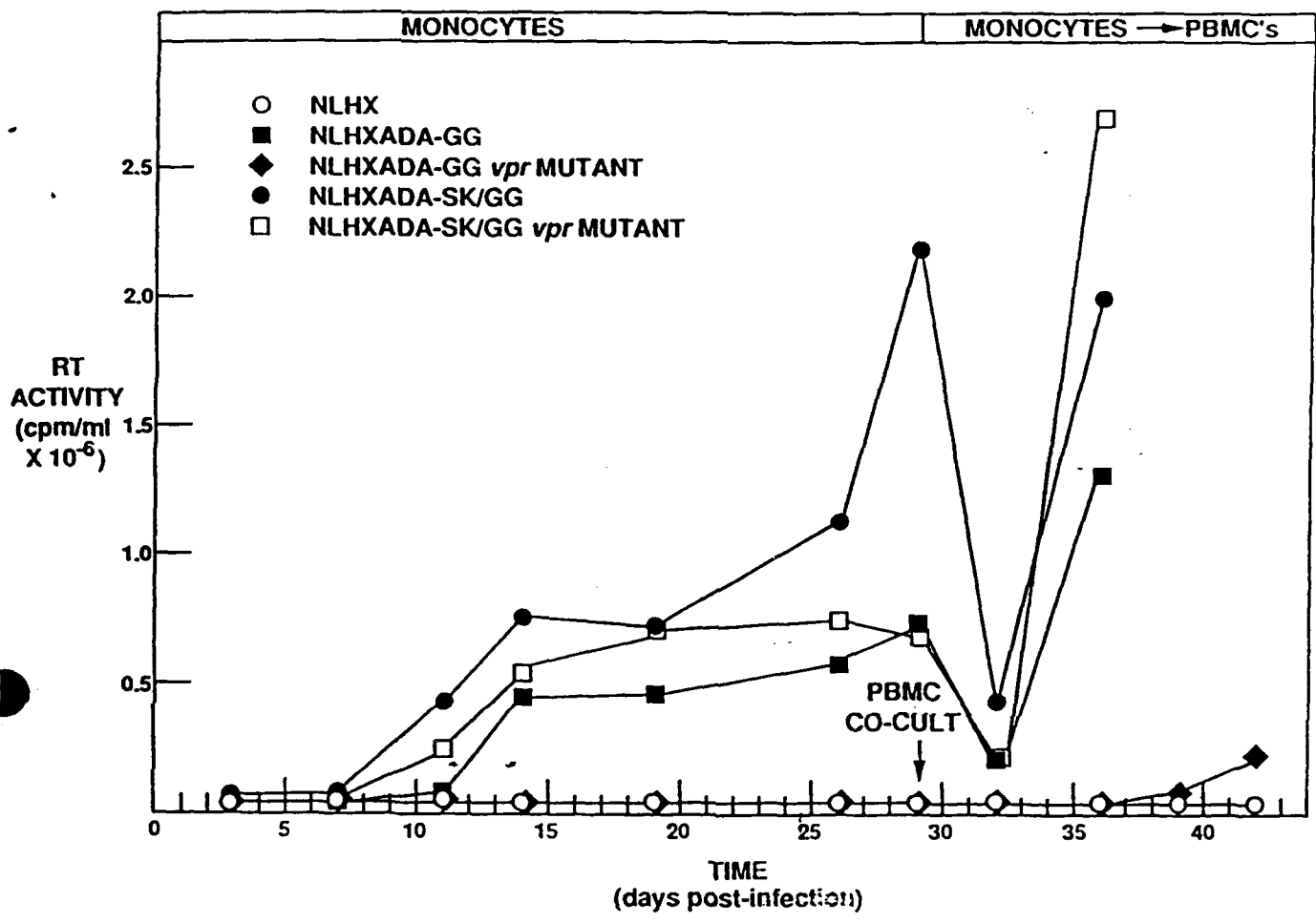


Fig. 8

D



1

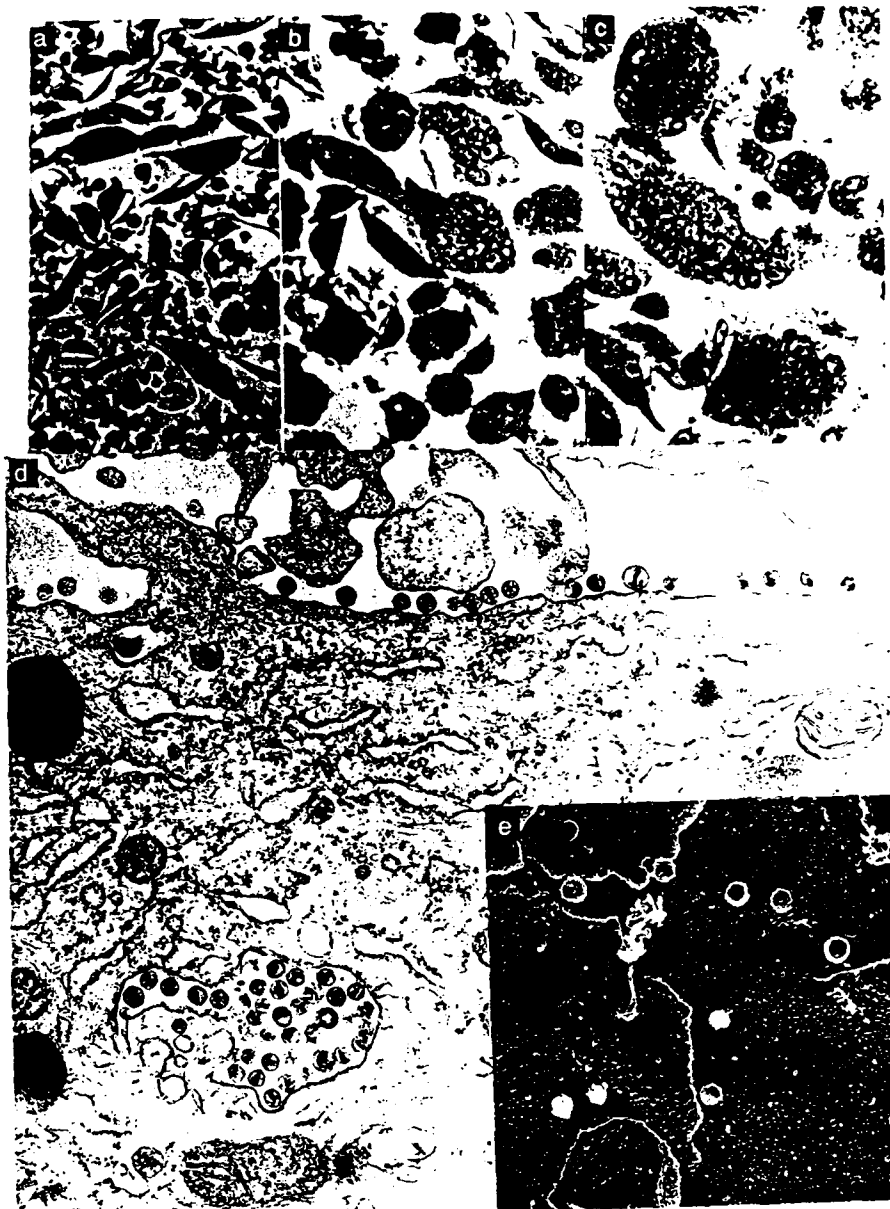


Fig. 10

These data suggest that vpr or vpu can convert silent infection of monocytes to productive infection. It is unclear whether or not these proteins are functioning at the same step or different steps. It is however, interesting that Vpu and Vpr have weak sequence similarity (Fig. 11), most notably in the C-terminus of Vpu and N-terminus of Vpr. It is also intriguing that sequence similarity of Vpr is noted with the HIV-2 Vpx protein (Fig. 11). Thus, it is not surprising that Vpx has been implicated as an important factor in monocyte infection by SIVmac, as well (Yu et al., 1991). In addition to the sequence similarity, notable similarities in hydrophilicity profile are depicted between these proteins (Fig. 11).

To determine whether vpu is the critical element with the SK fragment that allows productive infection, we have constructed site-directed mutations which place an initiator methionine codon into the vpu gene of HXB2, in clones NLHX-ADA-GG and NLHXADA-GP with or without the vpr mutation. This will provide definitive data as to whether vpu is the critical sequence within this DNA fragment for productive monocyte infection.

We have also begun to examine the role of the HIV-2 vpr gene in the presence or absence of the vpx gene in CEM lymphoid cells, primary lymphocytes, primary monocytes, or HeLa/T4 cells. For this purpose, we have utilized three clones previously constructed: ES with intact vpr and vpx genes, MX1+62 with mutations in vpx, and MR7 with a mutation in vpr. We constructed a new clone with both the vpr and vpx mutations, designated MR/MX. Thus far, no difference was noted in the presence or absence of an intact vpr gene. However, we have yet to demonstrate that our HIV-2 clone is capable of infecting monocytes. Cocultivation experiments with lymphocytes are underway to examine whether silent infection may occur.

h) Determine mechanism of action of Vpr in enhancing HIV-1 infectivity and/or replication in MT4 lymphoid cells

The contract application describes 3-10-fold differences in the ability of HIV-1 clones with or without vpr to replicate in HTLV-I infected cell lines, MT4 and MT2. However, the much more dramatic differences in the presence of vpr in monocytes, and the particular relevance of monocytes to disease pathogenesis, suggested to us that much greater emphasis should be placed on examining the mechanism of action of Vpr in monocytes than in MT4 cells. Thus, the same analyses described in the original contract application for MT4 cells studies are utilized for studies of Vpr in monocytes. Additional studies in MT4 cells will still be performed, but these studies will take a lower priority to pursuing the much more exciting monocyte infection studies.

Vpu 39 ..IDRLIDRITERAEDSGNESEGDQEE LSALVEMGHAPWDVDDL

Vpr 1 MEAQAPEDQGPQRE PYNEWTLELLEELKSEAVRHFR IWLHNLGQ HIYETYGDTWAGVEAIRILQQLFIHFRIGC...

Vpx 1 MTDPRETVPPGNSGEETIGEAFAWLNRTVEAINREAVNHLPRELIFQVWQRSWRYWHDEQGMSESYTKYRYLCII QKAVYMHVRKGC...

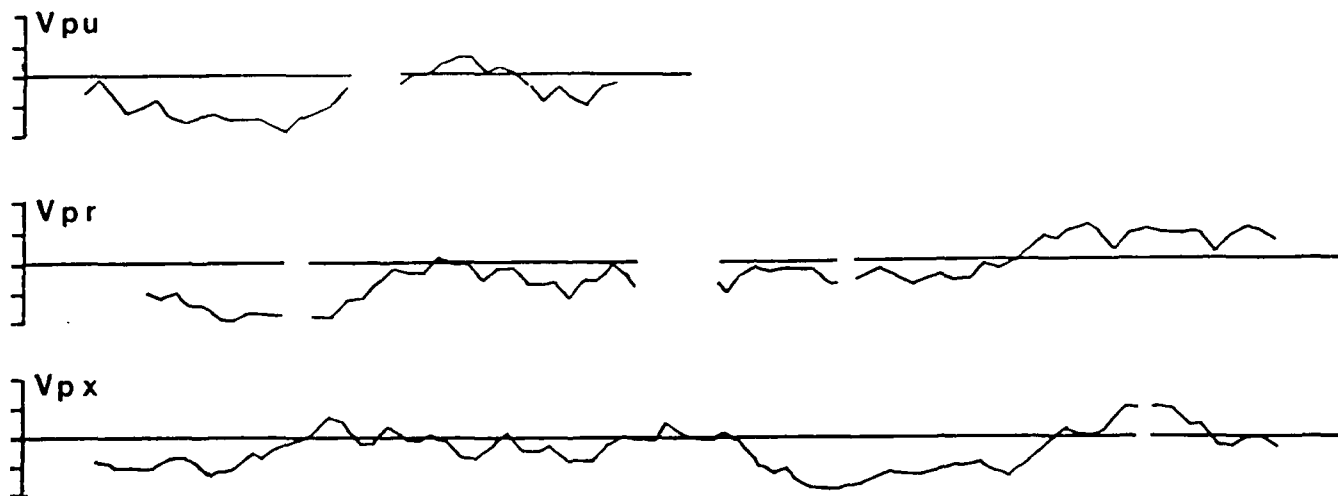


Fig. 11

i) Determine effects of Vpr on HIV-1 infectivity

Initial experiments have been performed to determine if Vpr affects the level of HIV-1 infection in monocytes. For this purpose, HIV-1 strains with or without the monocyte-tropic envelope determinant, vpr, and/or vpu were expressed from COS-7 cells and used to infect monocytes or lymphocytes. One day after infection, cellular DNA was harvested and quantitative PCR was performed with primers that were designed to detect the earliest reverse transcriptase products, i.e. R and U5 sequences. Controls were performed with heat-treated virus in order to insure that signals were not due to contaminating DNA in the virus preparations. These data demonstrated that the monocyte-tropism envelope determinant was essential for infection. Clone NIH3 gave rise to no detectable viral DNA synthesis in monocytes, whereas viral DNA could be detected in primary lymphocytes. Similar levels of viral DNA were seen in the presence of the monocyte-tropism envelope determinants in the presence or absence of vpr or vpu determinants. This experiment must be confirmed with other virus preparations and isolates. However, it suggests that Vpr and Vpu function at a step distinct from that of the envelope determinant after the initiation of reverse transcription. If similar findings are found in additional experiments of this type, other steps in virus replication will be examined in the presence or absence of the Vpr and Vpu determinants.

ii) Determine effects of Vpr on HIV-1 production

Studies of the effects of Vpr on HIV-1 production from monocytes have not yet been initiated, and will await the results of 1.h.i). If the results described above for HIV-1 DNA levels in monocytes are confirmed, we will repeat the assays examining HIV-1 RNA levels to assess whether Vpu and/or Vpr and affecting steps in the replication cycle between these two points (i.e. completion of reverse transcriptase, integration, transcription, RNA transport, RNA stability). Other studies will include those examining HIV-1 protein expression in infected monocytes by Western blot analysis and immunofluorescence.

We have examined by transmission electron microscopy monocytes infected productively or silently by a number of the virus strains above. No virus particles were detected in silently infected monocytes. Thus, either the replication cycle is aborted prior to the step of virus assembly, or the level of expression was too low to be detected by electron microscopy.

iii) Determine if Vpr effects can be complemented in trans

We have constructed SV40 expression plasmids to express HIV-1 Vpr or HIV-2 Vpx. An experiment is underway to determine if 1) the levels of the respective proteins expressed from cells transfected with each of these plasmids, and 2) these plasmids can complement defects found with HIV-2 expression from HeLa/T4 cells infected with vpx- virus, or with HIV-1 expression from primary human monocytes infected with vpr- virus.

iv) Determine if Vpr effects are independent of other regulatory proteins

We have not yet initiated experiments to assess whether defects in Vpr expression affect the expression of other regulatory proteins, i.e. Vpu, Nef, or Vif.

v) Determine the structure-function relationships of Vpr

To assess structure-function relationships of Vpr for productive infection of monocytes, we chose to first examine the C-terminal sequences that are highly conserved among different human and simian lentiviruses:

64	70	80	90	96	Vpr amino acid residue
**	** ** *	****			Residues conserved with HIV-2 & SIV Vprs
...IQQLFIHFRIGCRHSRIGVITQRRARNGASRS					HIV-1 NL4-3 Vpr protein

The following mutations have been constructed:

- C76 - Cysteine residue at residue 76 was converted to serine. It was of particular interest to determine if this highly conserved residue may be involved in dimer formation
- CRst- Vpr protein is truncated to 78 amino acid by frameshift at the Sal I site after residue 77. Cysteine residue 76 is left intact.
- SRIG- Residues 79-82 are deleted
- LF - Residues 68-69 are deleted
- LQ - Residues 64-65 are deleted
- F65 - Vpr product is truncated to a 65 amino acid protein due to a frameshift mutation, but an additional 21 amino acids are added to the C-terminus
- V65 - Vpr product is truncated to a 65 amino acid protein by the addition of a termination codon
- R2 - Vpr product is terminated at codon 2 by site-directed mutagenesis introducing a termination codon

Each of these clones has been constructed in NLHXADA-GP. All give rise to virus capable of replicating in primary lymphocytes. Studies are underway to assess their replication properties in monocytes.

The vpr gene of each of these mutants was cloned using PCR based technology into plasmid pTM3. This plasmid has a promoter for T7 polymerase and an EMC leader sequence to allow efficient translation in the absence of RNA capping. We have demonstrated that NL96 (parental Vpr protein), C76, CRst, and SRIG can be translated efficiently in reticulocyte lysates, labeled with [3H]-leucine, and specifically recognizes our anti-Vpr antisera (see 1)a). The V65 Vpr product could not be synthesized in reticulocyte lysates. We suspect that this molecule is highly unstable. The other forms of Vpr have not yet been examined in the reticulocyte lysate system. Studies of the electrophoretic mobility of these Vpr proteins in the presence or absence of 2-mercaptoethanol demonstrated no dif-

ference in the mobility of C76 and NL96, suggesting that disulfide-linked dimers are not formed.

We have also transfected these clones into BSC40 cells and infected with a recombinant vaccinia virus (VV) expressing T7 polymerase (VVT7). [3H]-Leucine labeled cells transfected with NL96 demonstrated expression of a similar sized protein to that expressed in reticulocyte lysates using the anti-Vpr antiserum. Studies of the Vpr mutants in BSC40 cells is underway.

i) Determine the role of Vpr in vivo with appropriate model systems

We have initiated studies in scid/hu mice with constructs NLHX, NLHXADA-GG, and NLHXADA-GG vpr mutant in collaboration with R. Markham (Johns Hopkins). The first experiment showed successful replication and recovery from inoculated mice with each virus. Quantitative assays are currently being developed using in situ hybridization and PCR.

We have deferred work in the rabbit model system as advised by our collaborators, Drs. Tom Folks and Michael Lairmore (CDC), since consistent results could not be reproduced with HIV-1 infection in this model system.

Studies of Vpr mutants in SIVmac239 are already underway in Dr. Desrosiers group.

j) Determine the role of Vpr in modulating disease in HIV-1 infected humans

In collaboration with Dr. Richard Markham (Johns Hopkins), we have obtained samples from seven patients followed over the course of several years with three PBMC samples from each patient at times when the CD4>1000, CD4=250-750, and CD4<200. PCR primers for amplifying RNA or DNA sequences from these samples are currently being evaluated.

2. To determine the function of viral protein X (Vpx)

a) To assess the effects of Vpx on replication and cytopathicity of HIV-2 in T lymphoid and monocytoid cells

i) To determine effects of Vpx on site of virus assembly in the cell

Studies on the effects of Vpx on site of virus assembly will be initiated in March, 1992, when Dr. Jiang Wang, an experience electron microscopist from Taiwan joins our laboratory.

ii) To determine the cellular localization of Vpx in HIV-2 infected cells

Immunogold electron microscopy to identify the cellular localization and viral localization of Vpx will be performed by Dr. Wang, beginning in March, 1992. For this purpose we will use HIV-2 infected cells, or cells transfected with Vpx expression clones. We have already constructed a clone that expresses Vpx from an SV40 promoter. We are currently cloning the HIV-2 Vpx gene into pTM3

for expression in cells infected with VVT7. In addition HIV-2 Vpr is also being cloned into pTM3.

iii) To determine if there are co- or post-translational modifications of Vpx

The clones described in the previous section will be used for these studies. In addition, studies will be performed in HIV-2 infected cells and cells transfected with Vpx expressed from the SV40 promoter.

d) To assess whether Vpx proviral mutants can be complemented in trans by a Vpx expression clone

These experiments are underway and are described in section 1.h.iii.

e) To determine structure-function relationships of Vpx

In addition to the studies of Vpx outlined above, a number of other clones are being constructed at this time to answer the following questions:

- i) Does Vpx binds HIV-2 RNA with specificity?
- ii) What HIV-2 viral components are required for packaging Vpx?

To examine Vpx binding to HIV-2 RNA, as noted previously, we are cloning HIV-2 vpx and vpr genes into pTM3 expression to allow expression in reticulocyte lysates. In addition, we are cloning the HIV-2 genome in both orientations into pGEM3ZF+. The latter plasmids will be used for in vitro transcription to synthesize sense and antisense forms of the HIV-2 genomic RNA. We will then examine binding of the reticulocyte lysate translated proteins to RNA by one or more of the following assays: Northwestern blots, filter binding assays, sucrose gradient sedimentation. If specific RNA binding can be detected, we will first localize the sequences required for binding in the HIV-2 RNA. Second, we will construct mutations in Vpx to determine which sequences are required for binding.

To investigate issues related to Vpx packaging, two approaches are currently underway. First, we are constructing clones of HIV-2 which are incapable of synthesizing envelope, by introduction of a linker within an Fsp I site in the env gene. This will be useful in asking whether envelope protein is required for Vpx packaging into virus particles. We are also constructing a packaging mutant of HIV-2. For this purpose, we are first subcloning the 5' Nar I fragment into a subclone. A deletion in the leader sequence expected to contain the packaging signal is being constructed through the use of PCR. We will then clone the segment with the deletion back into the full clone, to determine if the RNA packaging signal is required for Vpx packaging. An additional way to examine whether RNA is required for Vpx packaging is to determine if virus particles with only Gag and Pol proteins can package Vpx. For this second approach, the gag and pol genes are being cloned in pTM3. This clone will then be transfected into BSC40 cells infected with VVT7. Co-transfection experiments with Vpx expression

clones will be performed to examine if Vpx can be packaged into virus particles expressed in this way. We will ultimately determine the minimal requirements for Vpx packaging into particles, and Vpx sequences required for packaging.

f) To assess effects of Vpx on replication and cytopathicity of SIV in T lymphoid and monocytoïd cells

We have focused our initial work on the HIV-2 Vpx protein. Studies of SIVmac Vpx protein will be done subsequently.

g) To assess the role of Vpx in vivo with animal model systems

Animal model system studies will await initial data with the HIV-1 viruses in scid/hu mice (see 1.i.) and the initial data with the HIV-2 virus studies outlined in this section.

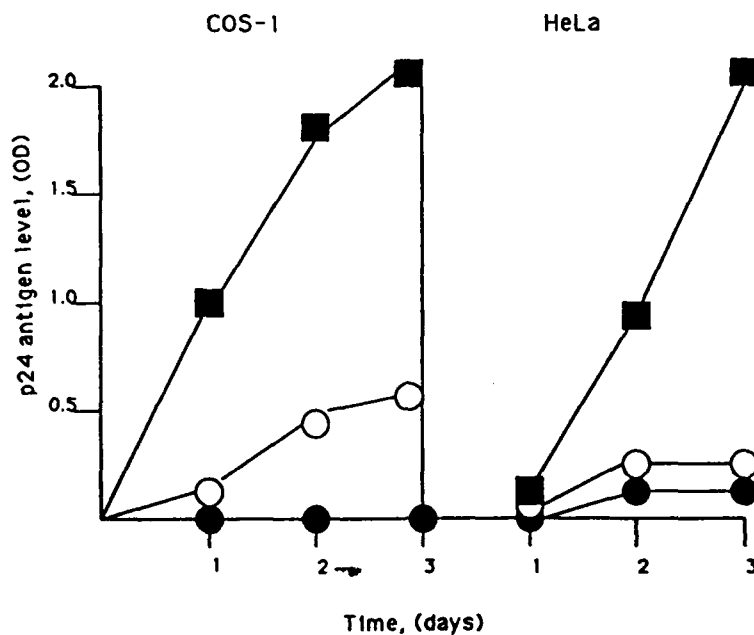
3. To determine the function and mechanism of action of negative factor (NEF)

a) To determine relative effects of HIV-1 Nef on viral transcription, degradation, and nuclear-cytoplasmic transport

The amino acid sequence alignment of Nef proteins from human and simian lentiviruses (Fig. 12) shows areas of conservation among these otherwise diverse protein sequences. Of note is the conservation of the myristoylation acceptor site encoded by the second codon of each nef gene. The protein kinase C phosphorylation acceptor site in the HIV-1 strains shown is not well conserved with those of other HIV-1 strains, HIV-2, or SIV strains. The second AUG codon at codon position 20 and the potential N-glycosylation site of the nef gene of HIV-1 strains is also not found in nef genes of HIV-2 and SIVs.

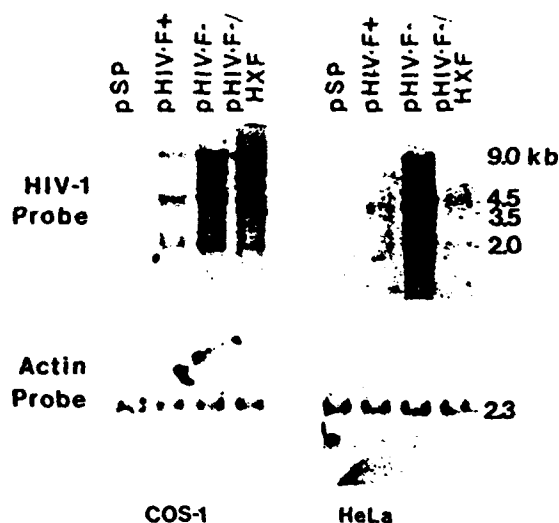
Expression in transfected COS-1 or HeLa cells of HIV-1 strains with defects in Nef was significantly higher than those of HIV-1 strains capable of encoding a full-length Nef protein (Fig. 13). A similar difference in levels of all three classes of viral RNA species was also identified in cells transfected with proviral clones with Nef compared to those with a truncated nef gene (Fig. 14). The suppressive effect of Nef on viral RNA levels was shown to be at the level of RNA transcription, as demonstrated in nuclear run-on assays (Fig. 15).

The work was confirmed and extended using SIVmac strain 102. The nef gene in this SIV strain is 38% similar to that of HIV-1 strain HXB2 (Fig. 16). A nef mutant clone (SIV BA) was constructed by PCR site-directed mutagenesis inserting a termination codon at position 40, without affecting the env coding sequence, and eliminating the Bam HI site (Fig. 17). COS cells were transfected with a control DNA lacking nef sequences, pSV2gpt (Fig. 18, lane 1), pSIV 102 (lane 2), or pSIV BA (lane 3), and then labeled with [35S]-methionine and [35S]-cysteine, and immunoprecipitated with an anti-SIV antiserum. The 32 kd Nef protein was detectable only in cells transfected with clone pSIV102 (indicated by *, Fig. 18). Gag proteins precipitated with the same antiserum from pSIV 102 and pSIV BA transfected cells are also indicated (g, Fig. 18).



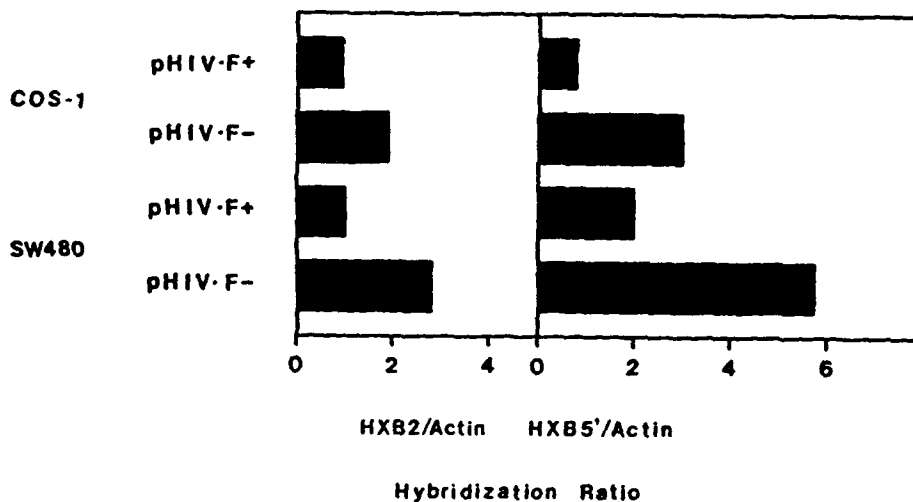
NEF decreases the level of p24 GAG antigen. Ten micrograms of pHIV F- (solid squares), pHIV F+ (open circles), or pSP (lacking HIV-1 sequences; closed circles) was transfected onto 50% confluent 100 mm plates of COS-1 or HeLa cells. Samples of cell culture media were tested for p24 antigen by an ELISA (DuPont). A sample of p24 (1 ng/ml) had an OD values of 0.82, and OD values were linear with protein concentration with the range of values reported. This experiment was repeated five times in COS-1 cells, and twice each in HeLa, HOS, and SW480 cells with similar results. Adapted with permission from Niederman et al. (1989).

Fig. 13



The HIV-1 *nef* product down-regulates the level of HIV-1 RNAs. Plates of COS-1 or HeLa cells were transfected with 10 micrograms of pHIV F+, pHIV F-, of pSP alone or together with an additional 30 micrograms of *nef* expression clone pHXF. RNA was harvested after 3 days, and Northern blot analysis was performed with 35 micrograms of RNA using Optiblot filters. Filters were hybridized with a full-length HIV-1 genome probe, or an actin probe. Reprinted with permission from Niederman et al. (1989).

Fig. 14



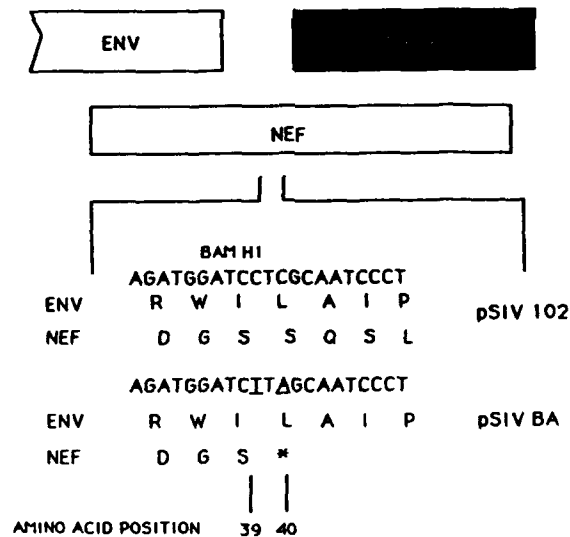
The HIV-1 *nef* product down-regulates the rate of synthesis of HIV-1 RNAs. Plates were transfected as described in Fig. 4. Nuclear run-on assays were performed and the [32P]-labeled transcripts were hybridized to 0.5 microgram aliquots of pHXB2 (HIV-1 nucleotides 1-9213), pHXB5' (HIV-1 nucleotides 222-5580), or actin. The ratio of hybridization to HIV-1 sequences compared to actin is shown. Reprinted with permission from Niederman et al. (1989).

Fig. 15

	Site of Myristoylation	PKC Phosphorylation Site		
HIV-1	NGC	KVKSSVIGVTV-REHRAEPAAD	CVC--AASRDLEKH	40
SIVmac	NGGATSKARSTKPGCDLRQKLLRARGETTCRLLGEVEDGSSQSGLGCLGCLSSSCEQ			58
HIV-1	CAITSS--NTAANNAACAVLEAQ	EEKVGFPVTRQVPLRPHTYKAA		84
SIVmac	KYNQQTNTTPWNPAREKIDCLAYRQNHDDIDEDDGLVGVSRPKVPLRAHTYKLA			117
HIV-1	VDLSHFLKEK	GCLEGLINSQRQDILD	LWIWNTQCYFFDMQNYTFCGPGIRYPLTFC	140
SIVmac	IDMSHFIKEK	GCLEGLIYTSARQRILD	HYLEKEEGCIIPDAQDTTSQPGIATYKTC	172
HIV-1	WRYKLVPVPEKLEEAANKGENTSLIMPVSLNGHDDPEREVLG-URFDSRLAFHNVARE			198
SIVmac	VLMLKLPVNVSDAEQDERHYLNQPAQTSKV--DQFUCEVLLANKSDPTLAYTYTAYV			231
HIV-1	LMPETFKNC			206
SIVmac	RYTPELEASQACQKRLKEC			250

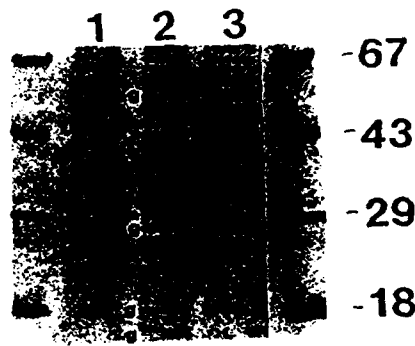
Amino acid alignment of the NEF proteins derived from HIV-1 (HXB2/3gpt [24, 26]) and SIV isolate 102 (derived from SIV MAC-251 [22]). Based on this alignment, there is a 38% sequence similarity between HIV-1 NEF and SIV NEF. Both forms of NEF may be myristoylated and share sequence similarity to the nucleotide binding domain of G proteins (boxed sequences).

Fig. 16



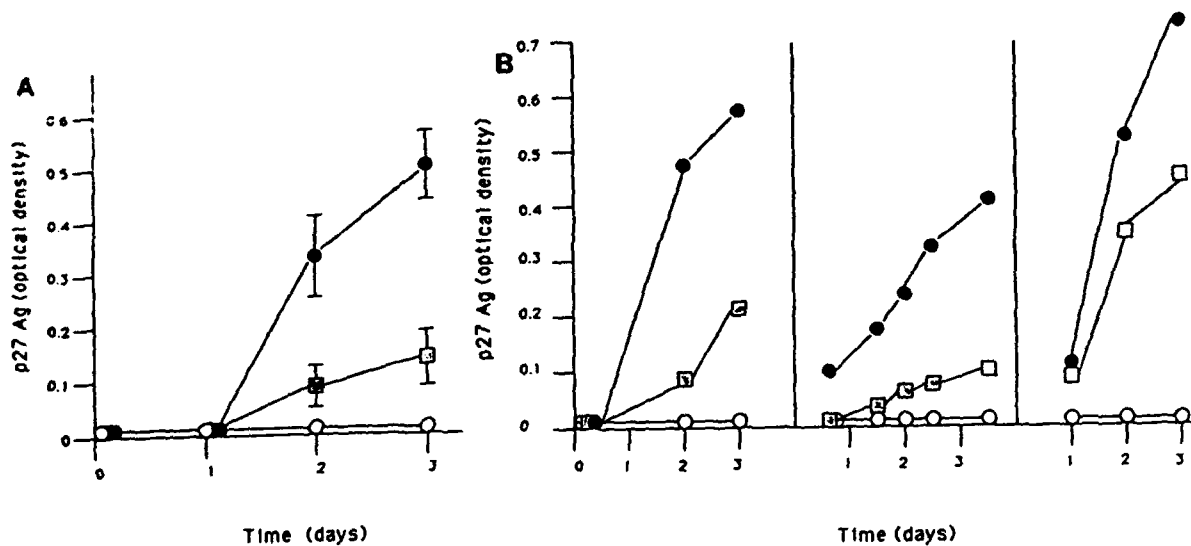
Construction of *nef* mutant clone pSIV BA from wild-type clone pSIV 102. A premature termination codon was engineered at codon 40 of the *nef* gene by using polymerase chain reaction site-directed mutagenesis with a synthetic oligonucleotide spanning the mutated region. The truncated NEF protein contains only the first 39 amino acids, but the ENV protein is unaffected by the mutation since *env* and *nef* lie in different reading frames.

Fig. 17



Expression of SIV NEF and GAG proteins. COS cells were transfected with 10 μ g of pSV2gpt (lane 1), pSIV 102 (lane 2), or pSIV BA (lane 3). At 48 h posttransfection, cells were labeled with [35 S]methionine and [35 S]cysteine and immunoprecipitated with serum from an infected macaque. Immunoprecipitated samples were then denatured and subjected to SDS-PAGE in a 7.5 to 20% gradient. *, NEF protein expressed in pSIV 102-transfected cells; g, precursor and processed gag proteins in pSIV 102- and pSIV BA-transfected cells.

Fig. 18



NEF downregulates SIV replication in transfected COS cells. In seven independent experiments, 10 μ g of either pSV2gpt, pSIV 102, or pSIV BA was transiently transfected into COS cells. An aliquot of the overlying medium was removed at the indicated times after transfection and assessed for solubilized p27^{core} core protein with (A) a Coulter SIV p27 ELISA kit or (B) an Abbott p24 HIV ELISA kit. (A) Averages of four experiments with error bars indicating 1 standard deviation from the mean. (B) Three different experiments. Transfection efficiency was monitored by cotransfecting pSV2CAT and measuring CAT activity; however, this parameter did not vary by more than 10% in any given experiment. For panel A, 1 optical density unit is approximately equal to an antigen concentration of 15 ng/ml. Symbols: ○, pSV2gpt; □, pSIV 102; ●, pSIV BA.

Fig. 19

Similar to the results with HIV-1, we found that in the presence of an intact nef gene, SIVmac expression was decreased 3-5-fold compared to that using the clone with a truncated nef gene (Fig. 19). Levels of all classes of viral RNAs were similarly depressed by Nef (Fig. 20 and Table 1). We noted depression of correctly initiated RNAs by Nef but no new RNA sites being utilized (Fig. 20). Two-three-fold decreases in the rate of RNA synthesis were also noted in the presence of Nef (Fig. 22), but no change in the stability of viral RNAs (Fig. 23).

b) To characterize Nef responsive sequences

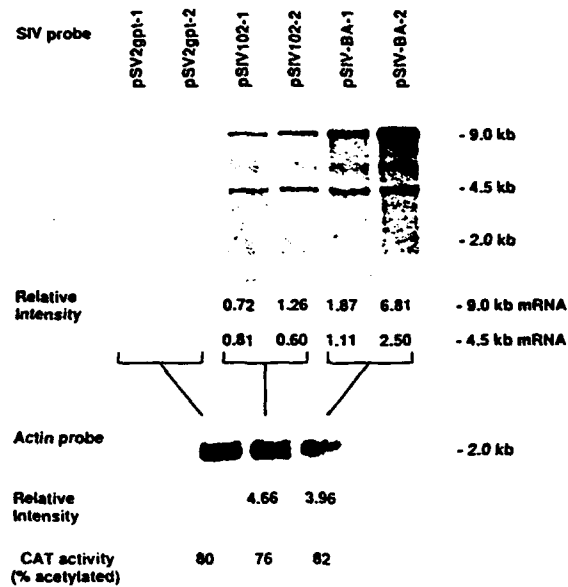
We initially attempted to characterize Nef responsive LTR sequences using indicator plasmids with various deletions of LTR sequences. However, inconsistent results were obtained. This was due to competing sequences present in the nef expression clone and the LTR-CAT constructs, the low level of transcriptional suppression (2-10-fold), and variation in the efficiency of transcription of different cell lines.

To overcome at least some of these difficulties, we are utilizing stably transfected and selected cell lines. In collaboration with Drs. V. Garcia (U. of Washington) and I. Chambers (Stanford University), we have obtained Jurkat, U937, HPBALL, and COS cells expressed HIV-1 Nef proteins. These clones will be transfected with HIV-1 LTR CAT constructs and control constructs to determine if there is an effect of Nef on expression. In addition, we have received from the AIDS Repository (NIAID) HeLa/T4, U937, and H9 cell lines stably transfected with HIV-LTR CAT. These cell lines will be transfected with our nef expression clones. In addition, in collaboration with Dr. John Majors (Washington University), we are constructing retrovirus vectors that express either Nef, Tat, or the HIV-1 LTR-beta galactosidase. We will assess effects of Nef on LTR activity by co-infection of the cells, and define critical elements in this case by in vivo DNA footprinting (see 3.c.ii.).

c) To characterize the mechanism of transcriptional suppression by Nef

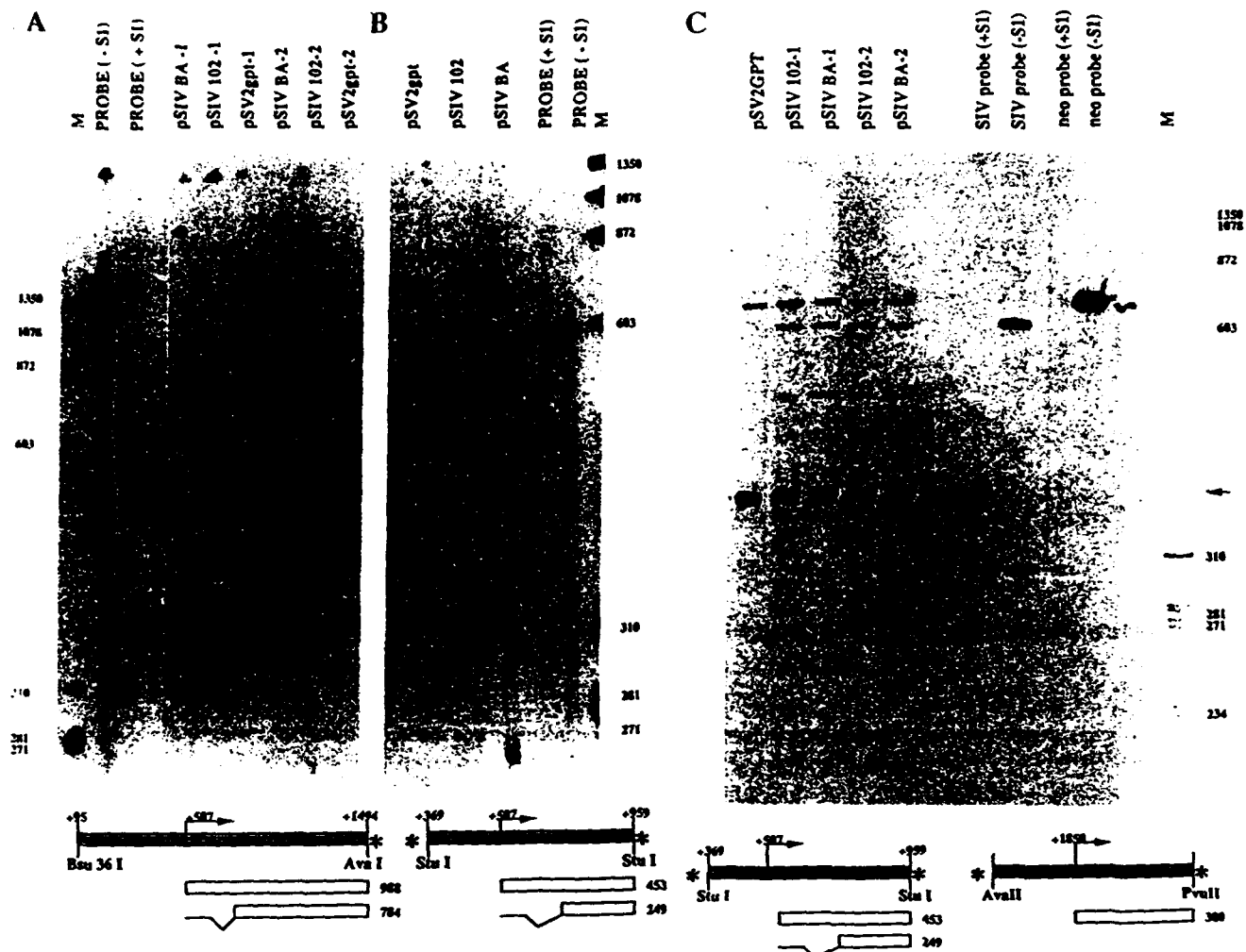
i. Gel retardation experiments

We have performed extensive gel retardation experiments over the last six months to define the responsive region of the LTR. For this purpose, we have used a wide range of different cell types transiently or stably transfected with nef expression plasmids as described in the previous section. In addition, monocytoid or lymphoid cell lines were examined in their basal state or in their activated state (e.g. LPS +/- PMA treated U937 cells, PMA +/- PHA +/- ionomycin treated Jurkat or HPBALL cells). Lastly, we have also prepared extracts from cells infected with different strains of HIV-1. For this analysis we have utilized a wide range of different labeled DNA fragments from the LTR and different oligonucleotides. Different parameters of the binding assay and competitor DNAs and oligonucleotides were examined.



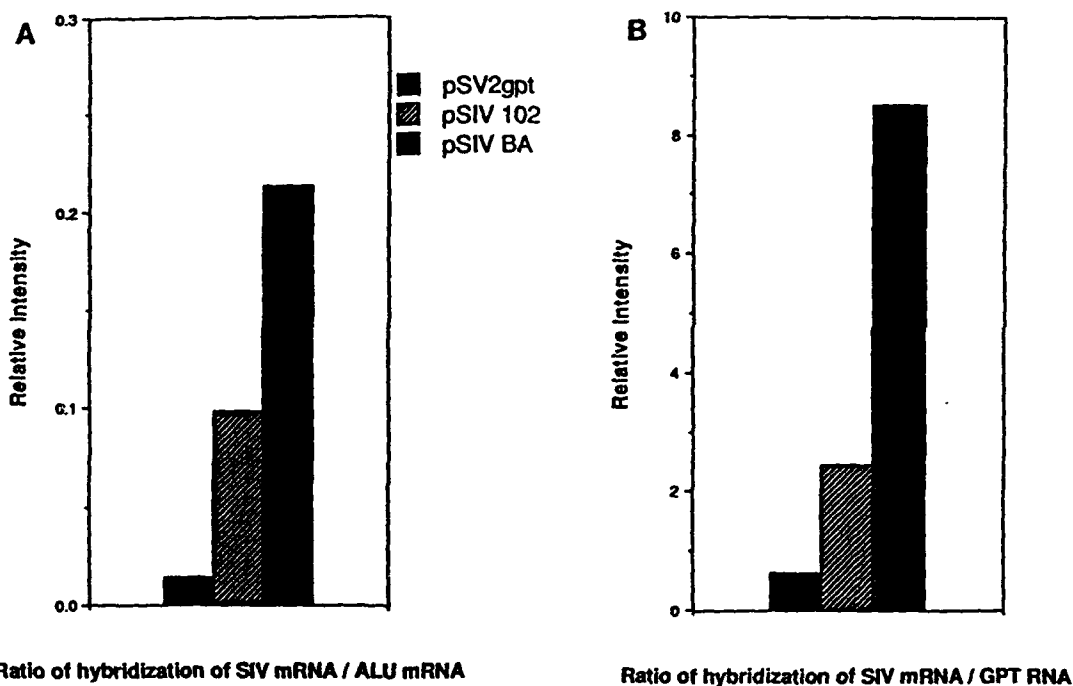
SIV NEF decreases viral mRNA accumulation. COS cells were transfected with 10 μ g of either pSV2gpt, pSIV 102, or pSIV BA and cotransfected with clone pSV2CAT to control for transfection efficiency. Total cellular RNA was isolated 3 days after transfection from 90% of the cells and subjected to Northern blot analysis. Protein extracts were prepared from the remaining 10% of the cells for CAT analysis. Hybridization was performed with hexamer-primed probes generated from the 3.5-kb *SacI* fragment from clone pSIV 102. In this experiment, duplicate transfections were performed. RNAs from experiments 1 and 2 were combined equally, subjected to Northern blot analysis, and hybridized to actin sequences that demonstrated that similar amounts of RNA were applied to the gel. CAT activities were similar within the linear range of analysis.

Fig. 20



SIV NEF decreases steady-state levels of SIV mRNA and does not affect the transcription start position. COS cells were transfected with proviral clones pSIV 102 and pSIV BA, and total cellular RNA was isolated 72 h later. RNA was annealed to either the *Bsu36I* (nt 94)-to-*AvaI* (nt 1494) fragment (A) or the 590-nt *StuI* fragment (B). RNA was annealed to the 590-nt *StuI* probe and the 655-nt *AvaII*-to-*PvuII* *neo* probe in panel C. Samples were then digested with S1 nuclease, and the protected, labeled DNA fragments were separated on denaturing PAGE (5% polyacrylamide). Symbols: , length of the probe; , region of protection; *, labeled sites. The numbers next to the empty boxes indicate the lengths of the protected fragments of the unspliced and spliced transcripts (29). The arrow over the striped box at nt 507 indicates the RNA CAP site. Panel A represents two independent experiments (1 and 2), panel B represents a third experiment, and panel C represents two additional independent experiments. Transfection efficiency, measured by cotransfecting pSV2CAT and assaying CAT activity (percent acetylation), was determined for all transfections. In part A, the mean CAT activity was $3.0\% \pm 0.4\%$ and $3.9\% \pm 0.7\%$; in part B, the activity was 3.9% and 3.4% ; and in part C, the mean activity was $70\% \pm 6\%$ and $69\% \pm 6\%$ for pSIV 102- and pSIV BA-transfected cells, respectively. In addition, the intensity of the protected *neo* probe (band indicated by arrow on right) was determined, and the value was used to adjust the relative concentration of viral mRNAs from transfected cells.

Fig. 21

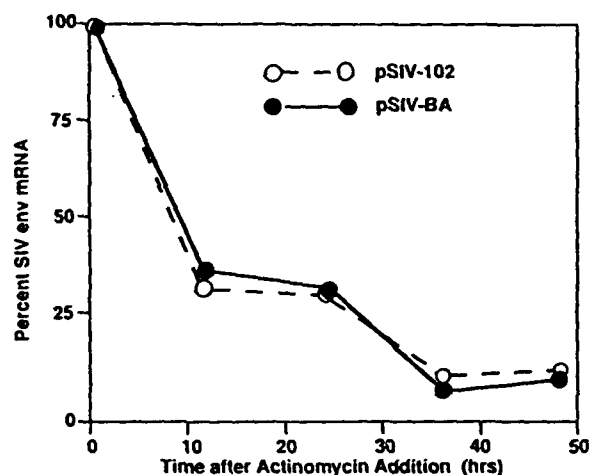
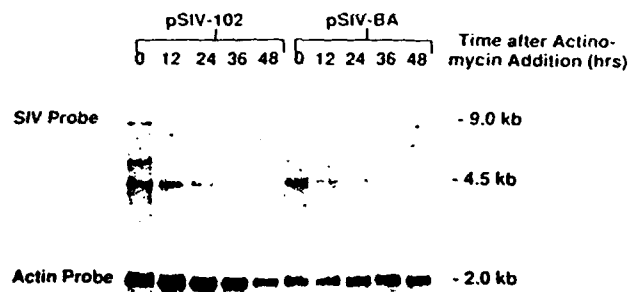


Ratio of hybridization of SIV mRNA / ALU mRNA

Ratio of hybridization of SIV mRNA / GPT RNA

SIV NEF decreases the rate of viral RNA synthesis. Nuclear run-on assays were performed with [α - 32 P]UTP (specific activity, 3,000 Ci/mmol) to label nuclei isolated from COS cells transfected with 10 μ g of pSV2gpt, pSIV 102, or pSIV BA. The nascent, labeled transcripts were hybridized to SIV sequences (pSIV 102), *AluI* repetitive sequences (pL33M), or *gpt* sequences (pSV2gpt) to control for the efficiency of labeling, RNA yield, and transfection efficiency. Hybridization intensity was measured by densitometry and is expressed as the ratio of hybridization to SIV sequences to that to *AluI* (A) or *gpt* (B) sequences.

Fig. 22



SIV NEF does not destabilize SIV mRNAs. COS cells (in eight 75-mm flasks) were transfected with either clone pSIV 102 or pSIV BA. At 24 h after transfection, the COS cells were harvested and pooled with similarly transfected cells and replated onto 150-mm plates. At 24 h after replating, the cells were incubated with dactinomycin for 0 to 48 h. At the indicated times, total cellular RNA was isolated and subjected to Northern blot analysis. The blot was first hybridized with the SIV probe as in the experiment shown in Fig. 4, exposed to film, stripped of RNA, and rehybridized to an actin probe as a control for the amount of RNA loaded on the gel. In this analysis, the stability of the 4.5-kb *env* mRNA was measured because it was the predominant species. At each time point, the calculated amount of SIV mRNA was determined by densitometry and the level of *env* mRNA was adjusted to the amount of actin mRNA.

Fig. 23

TABLE 1. SIV NEF downregulates viral mRNA levels^a

Expt	Proviral pSIV clone	Ratio ^b	mRNA suppression			CAT activity ^c (%)	Analysis
			SIV	Actin	neo		
1	102	2.7	0.9	1.8	8.9	64	Slot blot
	BA		2.1	1.4	6.1	74	
	102		1.3	2.3	7.2	77	
	BA		2.5	1.8	9.1	63	
2	102	1.9	2.1	1.1	7.2	44	Slot blot
	BA		2.9	1.3	9.7	50	
	102		2.4	1.5	12	49	
	BA		2.6	0.7	8.8	50	
	102		2.2	1.8	7.7	39	
	BA		1.9	0.7	7.3	50	
3	102	2.6	1.8	2.7	ND ^d	ND	Slot blot
	BA		3.6	2.8	ND	ND	
	102		3.0	1.6	ND	ND	
	BA		8.8	1.4	ND	ND	
4	102	1.7	1.4	5.3	ND	ND	Slot blot
	BA		2.3	4.8	ND	ND	
5	102	4.0	0.8	4.7	ND	76	Northern
	BA		1.5	4.0	ND	82	
	102		0.9	4.7	ND	76	
	BA		4.7	4.0	ND	82	
6	102	5.8	1.4	5.1	ND	3.4	S1 nuclease
	BA		6.2	3.9	ND	3.2	
	102		1.0	4.2	ND	2.5	
	BA		6.3	4.7	ND	4.6	
	102		0.6	5.1	ND	3.9	
7	102	3.5	3.5	5.0	ND	ND	Northern
	BA		7.1	2.9	ND	ND	
8	102	1.9	2.4	6.7	ND	ND	Northern
	BA		2.2	3.3	ND	ND	
9	102	2.7	1.0	0.9 ^e	ND	ND	Run-on
	BA		2.9	1.0 ^e	ND	ND	
10	102	2.6	1.5	1.4 ^e	ND	ND	Run-on
	BA		2.0	0.7 ^e	ND	ND	

^a This table summarizes the data from 10 separate experiments and includes 17 different matched pairs of densitometry scans of SIV, actin, or *neo* mRNAs (expressed as relative areas under each curve) from pSIV 102- and pSIV BA-transfected COS cells. In all cases, COS cells were transfected with 10 μ g of proviral DNA per 100-mm plates. These data include mRNAs that were analyzed by slot blot and Northern blot hybridization, S1 nuclease protection assays, and nuclear run-on assays. We calculated the level of viral mRNA suppression for each of the 17 matched pairs of pSIV 102 and pSIV BA values in order of the pairs from the top of the table downward. We found that the mean suppression of NEF on viral mRNA levels was 3.2-fold \pm 1.6-fold, where 1.6 represents the standard deviation. In addition, the 95% confidence intervals (2.4 and 4.0) indicate that the mean of 17 random matched pairs will lie between 2.4- and 4.0-fold 95% of the time.

^b Average ratio of the SIV densitometry values of pSIV BA divided by those of pSIV 102 multiplied by the ratio of actin densitometry values of pSIV 102 divided by those of pSIV BA-transfected cells.

^c CAT activity (measured as percent conversion to acetylated products) of a pSV2CAT plasmid that was cotransfected with the SIV proviral clones.

^d ND, Not done.

^e In the nuclear run-on assays, hybridization to *AluI* sequences served as a control for viral RNA concentration determinations instead of actin sequences.

The clear and consistent finding from these studies was that Nef inhibits an inducible nuclear factor from binding to the NFkappaB binding site. The binding is competed by a wild type NFkappaB binding site oligonucleotide but not a mutant oligonucleotide. No inhibition was seen with an SP1 binding site oligonucleotide.

ii. DNA footprinting studies

After identifying the NFkappaB binding site as a site for Nef activity, we will begin shortly DNA footprinting studies to confirm these findings, and to identify the precise positions of protein contacts. We will use both the HIV-1 LTR and IL2 promoter for these studies, as well as control promoters.

iii. In vitro transcription studies

We have not yet initiated the in vitro transcription studies, but these will be begin shortly.

d) To determine the role of phosphorylation, GTP binding, GTPase activity, and myristoylation acceptor activity on Nef activity

We have constructed a myristoylation acceptor mutant of Nef in a full proviral clone and a Nef expression vector. Studies are underway with this mutant, to examine its effect of virus replication in lymphoid cell lines, and primary lymphocytes.

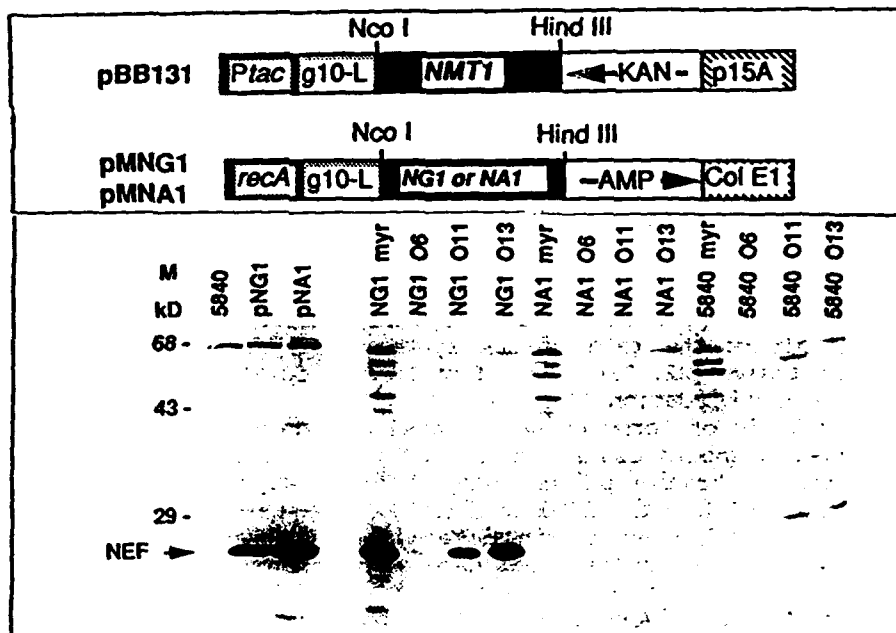
We have cloned nef genes with or without the myristoylation acceptor mutation (NG1 or NA1, respectively) into a bacterial expression clone, and co-expressed these proteins with yeast N-myristoyl transferase.

The data in Fig. 24 demonstrates that Nef can be expressed in these bacteria (left hand side) with or without the myristoylation acceptor. Only the NG1 Nef protein can be labeled with [3H]-myristic acid, or [3H]-analogs of myristic acid (O11 or O13). No labeling was seen with the [3H]-O6 analog. These myristoylated or non-myristoylated forms of Nef expressed in E. coli will be used to identify cellular receptors for Nef.

We have also cloned the myristoylation acceptor mutant and parental forms of nef into the pTM3 vector to allow expression in reticulocyte lysates and in VMT7 infected cells. In addition, these clones will be used for site-directed mutagenesis to construct clones with the protein kinase C acceptor mutation with or without the myristoylation acceptor mutation.

Oligonucleotides for the other mutations described in the original contract application have been synthesized. In addition, the C-terminal deletion mutants of Nef have also been constructed.

Preliminary studies on cellular localization of Nef have demonstrated an almost exclusive cytosolic localization on biochemical fractionation studies when using HIV-IIIB Nef proteins (Fig. 25). We are examining the cellular localization of other Nef variants expressed from other HIV-1 strains, and will examine the role of myristoylation and phosphorylation in cellular localization, using



Co-expression of *S. cerevisiae* N-myristoyl transferase and HIV-1 *nef* in *E. coli*. The dual plasmid expression system is shown in the top panel. The *S. cerevisiae* N-myristoyl transferase (NMT) is expressed from plasmid pBB131 utilizing an IPTG-inducible promoter, *Ptac*. Forms of Nef with a codon 2 glycine (pMNG1) or alanine (pMNA1) are expressed utilizing a nalidixic acid-inducible promoter, *recA*. The bottom panel shows co-expression of NMT and HIV-1 Nef in *E. coli* (50 μ g of total protein) labeled with [3 H]myristic acid, or [3 H]analog, and detected by immunoblot (left), or immunoprecipitation (right) with rabbit anti-Nef antiserum. Reprinted with permission from ref. 21.

Fig. 24



Cellular localization of the HIV-1 Nef Protein.

A) Immunoblot detection with a monoclonal anti-p24 antibody of HIV-1 Pr55gag and p24 in H9/IIIB cells after incubation with media alone (C), 0.1% ethanol (E), 40 μ M dodecanoic acid (C10), 40 μ M 12-methoxydodecanoic acid (O13), or 5 μ M azidothymidine (AZT). B) Immunoprecipitation with a human antiserum of HIV-1 proteins in 100,000 \times g soluble (S) or pellet (P) fractions of H9/IIIB cells metabolically labeled with [3 H]myristic acid, [3 H]O13, or [3 H]O6. The intact Pr55gag is indicated by an asterisk. C) Immunoprecipitation with rabbit anti-Nef antiserum of HIV Nef species from the same fraction as in panel B. The results shown in panels A-C are representative of those obtain in three independent experiments. Reprinted with permission

Fig. 25

the expression clones described above.

Fig. 26 provides a summary of our working model of the structure and function of Nef. It shows the protein localized on the inner surface of the plasma membrane through myristic acid linkage attached to glycine encoded by the second codon. Protein kinase C is capable of phosphorylating threonine residue 15. Residues 95-100 may play a role in nucleotide binding, and may lead to phosphorylation of Nef-responsive factors (NRF) or lead to autophosphorylation. A cascade may be induced by NRF that causes transcriptional suppression of the HIV-1 promoter.

e) To determine effects of Nef on cellular proteins including those which may modulate HIV-1 infectivity or replication

To examine the role of Nef on cellular proteins, we have taken two different approaches. We are examining effects on IL2 synthesis at the transcriptional level in experiments outlined above. We are examining effects of Nef on CD4 cell surface expression, in BSC40 cells co-infected with vaccinia virus expressing Nef or CD4. Effects of Nef on other cellular proteins will be examined with the stably transfected cell lines and the retrovirus expression clones that are under construction. Lastly, we are searching for Nef-binding proteins by several different assays. Most notably, we are currently screening a lambda gt11 cDNA expression library with iodinated Nef to detect Nef-binding proteins. Additional assays have utilized Sepharose columns to which we have bound purified Nef protein. This work is in progress.

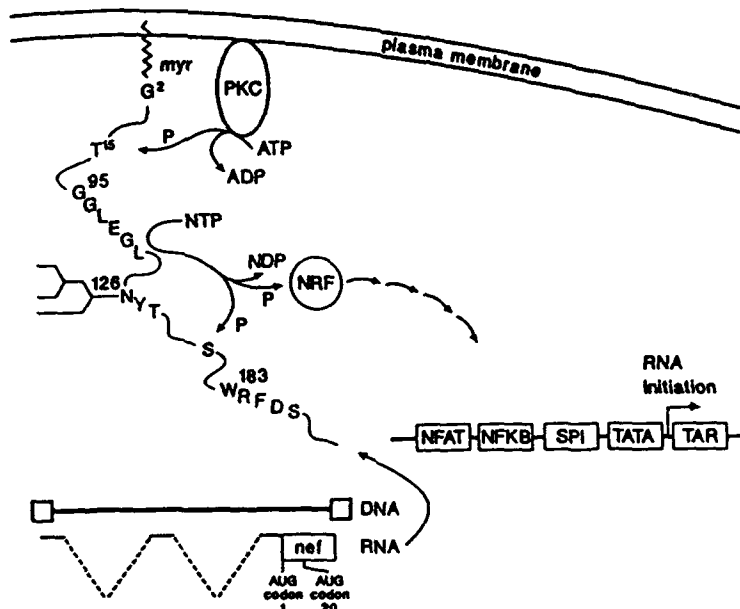
f) To determine the role of Nef in HIV-2 and SIV replication

We have described extensive work on the effects of Nef on SIV replication. Unfortunately, the SIVmac 102 strain utilized in the previous experiments is poorly infectious for most T cells. Therefore, we have utilized SIVmac239, the pathogenic SIV strain with a mutation in Nef, and constructed a Nef+ clone in which we have removed the termination codon. Experiments with these Nef- and Nef+ viruses are underway.

Studies of HIV-2 nef have not been initiated since the HIV-2rod strain that we have utilized has a large deletion in the nef gene. We have corrected this defect by addition of sequences from a cDNA clone, but we have not yet completed sequence analysis to confirm that the nef gene is now intact.

g) To determine the role of Nef in vivo with animal model systems

The SIVmac239 clones used for our analysis of Nef have already been studied by Desrosiers and colleagues in rhesus macaques, and these investigators demonstrated that Nef was critical for pathogenicity. Our studies in vitro with those virus strains should provide important information in explaining this finding. One possibility is that Nef is required for establishment of a state of "latency" in vivo, and that in the absence of Nef, infected cells are rapidly lost due to lysis or immune clearance.



. Structure and function of Nef. Shown in the lower left hand portion is a multiply sliced *nef* mRNA expressed from HIV-1 proviral DNA. Protein products are initiated at both the first and second AUG codons. The larger protein product is shown as a protein myristoylated at the glycine residue encoded from codon 2 of the Nef precursor (G2), possibly mediating binding to cellular membranes under certain conditions. Threonine at position 15 (T15) is phosphorylated by protein kinase C. A potential nucleotide binding domain is shown near residues 95-100. Cleavage of nucleotide triphosphate (NTP) may lead to autophosphorylation at a serine residue (S), as well as phosphorylation of a Nef-responsive factor (NRF) that mediates Nef activity in the nucleus. The asparagine at position 126 is glycosylated, and sequences near residue 183 may mediate GTPase activity (10). Potential sites for action of Nef on LTR activity are shown at the lower right. Reprinted with permission.

Fig. 26

Studies in scid-hu mice with the HIV-1 clones will be performed after the initial studies outlined in 1.i. are completed. We expect that these experiments will be initiated within the next 3-6 months.

h. To determine the role of Nef in modulating manifestations of HIV-1 infection in humans

We have used PBMCs from over the full course of disease of individuals described in 1.j. We have obtained the first 20 nef clones and preliminary sequence analysis has been initiated. We will obtain and sequence at least 8 clones from each of 3 samples from at least 4 of these patients, or about 100 clones in all. Functional studies on selected clones will be based on the sequence data.

i. To determine the therapeutic role of Nef in down-regulating HIV-1

Since Nef has been shown to be a pathogenic factor in SIVmac in rhesus macaques, our goal in this regard is altered. Nevertheless, we are in the midst of constructing the retroviral expression clones for Nef that would be useful for a wide range of studies, including down-regulation of HIV-1.

(7) CONCLUSIONS

In conclusion, in the first year of this contract significant progress was made both in development of necessary reagents, experimental assays, as well as in several very interesting novel findings.

With respect to Vpr, we have made a useful polyclonal antibody to HIV-1 Vpr and obtained antibody to HIV-2 Vpr. We have constructed a very interesting set of mutations in Vpr with alterations in the carboxyl terminus, altering specifically residues that are highly conserved in Vpr proteins of HIV-1, HIV-2, and SIVmac. Clones expressing HIV-2 Vpr are also currently being constructed. We have expressed parental and mutant Vpr proteins in reticulocyte lysates, using a vaccinia expression system, and in HIV-1 in cell lines and primary lymphocytes and monocytes. Cell localization experiments with these Vpr proteins have been initiated. Perhaps the most interesting finding, however, is that we have found that Vpr is very important for the ability of HIV-1 to productively infect monocytes. This requirement, however, can be replaced by Vpu. Additional mechanistic studies and animal model studies are focusing on further analysis of this very interesting finding.

With respect to Vpx, we have inoculated rabbits with a recombinant Vpx protein for antibody development. We have begun construction of Vpx expression clones for studies of cell localization, post-translational modification, and RNA binding studies. We have begun construction of env- and psi- clones to decipher the requirements for Vpx packaging. We have also found Vpx important for infection of HeLa/T4 cells. The mechanism for this effect is currently being studied.

With respect to Nef, we have obtained polyclonal antibodies to HIV-1 Nef. We have identified a critical step in Nef activity in both HIV-1 and SIVmac at the level of transcriptional initiation. We have identified through gel retardation experiments, a factor that binds to the NFkappaB binding element whose expression or binding activity is inhibited by Nef. We have constructed myristoylation acceptor mutants of Nef, and demonstrated defective myristoylation in an E. coli expression system. Parental, truncated, and myristoylation acceptor mutant Nef expression clones for reticulocyte lysates, recombinant vaccinia virus expression, or HIV-1 virus expression have been completed. In addition, we have constructed Nef mutations in several other HIV-1 strains and the pathogenic strain of SIVmac239 for further mechanistic studies.

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(9) APPENDIX

Publications

Manuscripts Submitted to Peer-Review Journals Supported by this Contract:

1. Westervelt P, Gendelman HE, Li Y, and Ratner L. A determinant within the HIV-1 gp120 envelope protein critical for infection of primary monocytes. Vaccines 91, Cold Spring Harbor Laboratory Press, pp. 71-76, 1991.
2. Niederman TMJ, Hu W, and Ratner L. Simian immunodeficiency virus negative factor (NEF) suppresses viral mRNA accumulation in COS cells. J. Virol. 65: 3538-3546, 1991.
3. Westervelt P, Gendelman HE, and Ratner L. Identification of a determinant within the HIV-1 surface envelope glycoprotein critical for productive infection of cultured primary monocytes. Proc. Natl. Acad. Sci. 88:3097-3101, 1991.
4. Bryant ML, Ratner L, Duronio RJ, Kishore NS, Adams SP, and Gordon JI. Incorporation of 12-methoxydodecanoate into the gag polyprotein precursor of HIV-1 inhibits its proteolytic processing as well as virus production in a chronically infected human lymphoid cell lines. Proc. Natl. Acad. Sci. 88:2055-2059, 1991.
5. Westervelt P, Henkel T, Trowbridge DB, Gendelman HE, and Ratner L. HIV-1 infection of primary monocytes: role of two distinct determinants -envelope and viral protein R. Manuscript in preparation.

Reviews Supported by this Contract:

1. Ratner L, Niederman TJ, Lozeron H, and Bryant M. Structure and function of the negative factor (NEF) of HIV and SIV. In Kumar, A., ed. *Advances in Molecular Biology and Targetted Treatment of AIDS*. Plenum Press, 1991.
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3. Bryant M and Ratner L. The biology and molecular biology of HIV-1. *Ped. Infect. Dis. J.*, In press.
4. Niederman T and Ratner L. Functional analysis of HIV-1 and SIV Nef proteins. *Research in Immunology*, In press.

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1. Westervelt P and Ratner. An HIV-1 gp120 envelope protein determinant critical for infection of primary monocytes. *Cold Spring Harbor Vaccine/AIDS Meeting*, 1990.
2. Westervelt P, Gendelman HE, and Ratner L. An HIV-1 gp120 envelope porteoin determinant critical for infection of primary monocytes. *Clin. Res.* 1990.
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4. Westervelt P, Trowbridge DB, Gendelman HE, and Ratner L. Regulation of silent and productive HIV-1 infection in primary monocytes by two distinct genetic determinants *Clin. Res.* 1991.
5. Westervelt P, Trowbridge DB, Gendelman HE, and Ratner L. Regulation of silent and productive HIV-1 infection in primary monocytes by two distinct genetic determinants. *Proc. 7th International Conference on AIDS*, Florence, Italy, 1991.
6. Ratner L, Westervelt P, Trowbridge DB, and Gendelman HE. Regulation of silent and productive HIV-1 infection in primary monocytes by two distinct genetic determinants. *American Society of Virology, 10th Annual Meeting*, Fort Collins, Colorado, 1991.
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8. Westervelt P, Trowbridge DE, Gendelman HE, and Ratner L. Regulation of silent and productive HIV-1 infection in primary monocytes by two distinct genetic determinants. Cold Spring RNA Tumor Virus Meeting, 1991.
9. Ratner L and Westervelt P. Molecular basis for HIV-1 tropism for monocytes:- interaction of envelope, vpr, and vpu proteins. American Society of Hematology, 1991.